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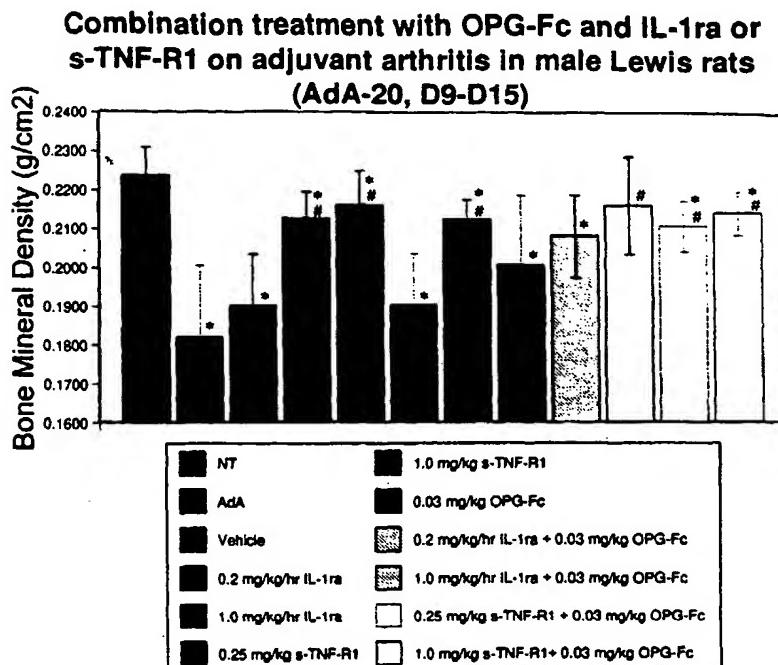
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(54) Title: COMBINATION THERAPY FOR CONDITIONS LEADING TO BONE LOSS



Paws from rats with adjuvant arthritis induced by 0.5mg mycobacteria in oil were analyzed by DEXA for BMD. Evaluation of BMD, a 20mm X 25mm was centered at the tibiotarsal region. (expt AdA-20 5/99, Amgen nb#22957 p88).

\* compared to normal, # compared to vehicle  
P < 0.05 Mann-Whitney U test.

(57) Abstract: The present invention discloses a novel secreted polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily and is involved in the regulation of bone metabolism. Also disclosed are nucleic acids encoding osteoprotegerin, polypeptides, recombinant vectors and host cells for expression, antibodies which bind OPG, and pharmaceutical compositions. The polypeptides are used to treat bone diseases characterized by increased resorption such as osteoporosis. Methods of treatment are described using the polypeptides in conjunction with various agents, including IL-1 inhibitors, TNF- $\alpha$  inhibitors, and serine protease inhibitors.

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**COMBINATION THERAPY FOR  
CONDITIONS LEADING TO BONE LOSS**

**Cross-Reference to Related Applications**

5        This application is a continuation-in-part of U.S. Ser. No. 09/350,670 filed July 9, 1999, which is a continuation-in-part (CIP) of U.S. Ser. No. 08/706,945, filed on September 3, 1996; which in turn is a CIP of U.S. Ser. No. 08/577,788, filed December 22, 1995. Each 10 of the foregoing applications is hereby incorporated by reference.

**Field of the Invention**

The invention relates generally to polypeptides involved in the regulation of bone metabolism. More 15 particularly, the invention relates to a novel polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily. The polypeptide is used to treat bone diseases characterized by increased bone loss such as 20 osteoporosis and arthritis.

**Background of the Invention**

Polypeptide growth factors and cytokines are secreted factors which signal a wide variety of changes in cell growth, differentiation, and metabolism, by 25 specifically binding to discrete, surface bound receptors. As a class of proteins, receptors vary in their structure and mode of signal transduction. They are characterized by having an extracellular domain that is involved in ligand binding, and cytoplasmic 30 domain which transmits an appropriate intracellular signal. Receptor expression patterns ultimately determine which cells will respond to a given ligand, while the structure of a given receptor dictates the cellular response induced by ligand binding. Receptors 35 have been shown to transmit intracellular signals via

their cytoplasmic domains by activating protein tyrosine, or protein serine/threonine phosphorylation (e.g., platelet derived growth factor receptor (PDGFR) or transforming growth factor- $\beta$  receptor-I (TGF $\beta$ R-I), 5 by stimulating G-protein activation (e.g.,  $\beta$ -adrenergic receptor), and by modulating associations with cytoplasmic signal transducing proteins (e.g., TNFR-I and Fas/APO) (Heldin, *Cell* 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) 10 superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, *et al.* *Cell* 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of 15 these receptors (Chen *et al.*, *Chemistry* 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNF $\alpha$ . (Goeddel *et al.* *Cold Spring Harbor Symp. Quart. Biol.* 51, 597-609 (1986); Nagata *et al.* *Science* 267, 20 1449-1456 (1995)). TNF $\alpha$  binds to distinct, but closely related receptors, TNFR-I and TNFR-II. TNF $\alpha$  produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler *et al.* *Ann. Rev. Biochem.* 57, 505-518 (1988)).

TNF $\alpha$  is believed to mediate acute and chronic inflammatory responses (Beutler *et al.* *Ann. Rev. Biochem.* 57, 505-508 (1988)). Systemic delivery of TNF $\alpha$  induces toxic shock and widespread tissue necrosis. 30 Because of this, TNF $\alpha$  may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda *et al.* *Cell* 75, 1169-1178 (1993)), is associated

with autoimmunity (Fisher *et al.* *Cell* 81, 935-946 (1995)), while overproduction of FasL may be implicated in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects 5 of many disease states, which suggests that agents that neutralize the activity of these ligands would have therapeutic value. Soluble TNFR-I receptors, and antibodies that bind TNF $\alpha$ , have been tested for their ability to neutralize systemic TNF $\alpha$  (Loetscher *et al.* 10 *Cancer Cells* 3(6), 221-226 (1991)). A naturally occurring form of a secreted TNFR-I mRNA was cloned, and its product tested for its ability to neutralize TNF $\alpha$  activity *in vitro* and *in vivo* (Kohno *et al.* *PNAS USA* 87, 8331-8335 (1990)). The ability of this protein 15 to neutralize TNF $\alpha$  suggests that soluble TNF receptors function to bind and clear TNF thereby blocking the cytotoxic effects on TNFR- bearing cells.

An object of the invention is to identify new 20 members of the TNFR superfamily. It is anticipated that new family members may be transmembrane proteins or soluble forms thereof comprising extracellular domains and lacking transmembrane and cytoplasmic domains. We have identified a new member of the TNFR superfamily 25 which encodes a secreted protein that is closely related to TNFR-II. By analogy to soluble TNFR-II, the TNFR-II related protein may negatively regulate the activity of its ligand, and thus may be useful in the treatment of certain human diseases.

A further object of this invention is new methods 30 of treatment of inflammatory diseases and medical conditions.

#### Summary of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily has been identified from a 35 fetal rat intestinal cDNA library. A full-length cDNA

clone was obtained and sequenced. Expression of the rat cDNA in a transgenic mouse revealed a marked increase in bone density, particularly in long bones, pelvic bone and vertebrae. The polypeptide encoded by the cDNA 5 is termed Osteoprotegerin (OPG) and plays a role in promoting bone accumulation.

The invention provides for nucleic acids encoding a polypeptide having at least one of the biological activities of OPG. Nucleic acids which hybridize to 10 nucleic acids encoding mouse, rat or human OPG as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO: 122), and 9C-9D (SEQ ID NO: 124) are also provided. Preferably, OPG is mammalian OPG and more preferably is 15 human OPG. Recombinant vectors and host cells expressing OPG are also encompassed as are methods of producing recombinant OPG. Antibodies or fragments thereof which specifically bind the polypeptide are also disclosed.

Methods of treating bone diseases are also 20 provided by the invention. The polypeptides are useful for preventing bone resorption and may be used to treat any condition resulting in bone loss such as osteoporosis, hypercalcemia, Paget's disease of bone, and bone loss due to rheumatoid arthritis or 25 osteomyelitis, and the like. Bone diseases may also be treated with anti-sense or gene therapy using nucleic acids of the invention. Pharmaceutical compositions comprising OPG nucleic acids and polypeptides are also encompassed.

30 The invention relates further to treatment of diseases using combination therapy. In particular, the novel polypeptides described herein may be used in conjunction with bone morphogenic proteins BMP-1 through BMP-12; TGF- $\beta$  and TGF- $\beta$  family members; IL-1 35 inhibitors; TNF- $\alpha$  inhibitors; parathyroid hormone and

analogs thereof; parathyroid related protein and  
analogs thereof; E series prostaglandins;  
bisphosphonates; bone-enhancing minerals; NSAIDs;  
immunosuppressants; serine protease inhibitors; IL-6  
5 inhibitors; IL-8 inhibitors (e.g., antibodies to IL-8);  
IL-18 inhibitors; ICE modulators; FGF-1 to FGF-10; FGF  
modulators; PAF antagonists; KGF, KGF-related  
molecules, or KGF modulators; MMP modulators; NOS  
modulators; modulators of glucocorticoid receptor;  
10 modulators of glutamate receptor; modulators of LPS  
levels; and noradrenaline and modulators and mimetics  
thereof.

**Description of the Figures**

Figure 1. A. FASTA analysis of novel EST LORF.  
15 Shown is the deduced FRI-1 amino acid sequence aligned  
to the human TNFR-II sequence. B. Profile analysis of  
the novel EST LORF shown is the deduced FRI-1 amino  
acid sequence aligned to the TNFR-profile. C.  
Structural view of TNFR superfamily indicating region  
20 which is homologous to the novel FRI-1.

Figure 2. Structure and sequence of full length  
rat OPG gene, a novel member of the TNFR superfamily.  
A. Map of pMOB-B1.1 insert. Box indicates position of  
LORF within the cDNA sequence (bold line). Black box  
25 indicates signal peptide, and gray ellipses indicate  
position of cysteine-rich repeat sequences. B, C.  
Nucleic acid and protein sequence of the Rat OPG cDNA.  
The predicted signal peptide is underlined, and  
potential sites of N-linked glycosylation are indicated  
30 in bold, underlined letters. D, E. Pileup sequence  
comparison (Wisconsin GCG Package, Version 8.1) of OPG  
with other members of the TNFR superfamily, fas (SEQ ID  
NO:128); tnfr1 (SEQ ID NO: 129); sfu-t2 (SEQ ID  
NO:130); tnfr2 (SEQ ID NO:131); cd40 (SEQ ID NO:132);  
35 osteo (SEQ ID NO:133); ngfr (SEQ ID NO:134); ox40 (SEQ  
ID NO:135); 41bb (SEQ ID NO:136).

Figure 3. PepPlot analysis (Wisconsin GCG Package, Version 8.1) of the predicted rat OPG sequence. A. Schematic representation of rat OPG showing hydrophobic (up) and hydrophilic (down) amino acids. Also shown are 5 basic (up) and acidic (down) amino acids. B. Display of amino acid residues that are beta-sheet forming (up) and beta-sheet breaking (down) as defined by Chou and Fasman (Adv. Enz. 47, 45-147 (1948)). C. Display of propensity measures for alpha-helix and beta-sheet 10 (Chou and Fasman, ibid). Curves above 1.00 show propensity for alpha-helix or beta-sheet structure. Structure may terminate in regions of protein where curves drop below 1.00. D. Display of residues that are alpha-forming (up) or alpha-breaking (down). E. Display 15 of portions of the protein sequence that resemble sequences typically found at the amino end of alpha and beta structures (Chou and Fasman, ibid). F. Display of portions of the protein sequence that resemble sequences typically found at the carboxyl end of alpha 20 and beta structures (Chou and Fasman, ibid). G. Display of portions of the proteins sequence typically found in turns (Chou and Fasman, ibid) H. Display of the helical hydrophobic moment (Eisenberg et al. Proc. Natl. Acad. Sci. USA 81, 140-144 (1984)) at each position in the 25 sequence. I. Display of average hydrophathy based upon Kyte and Doolittle (J. Mol. Biol. 157, 105-132 (1982)) and Goldman et al. (reviewed in Ann. Rev. Biophys. Biophys. Chem. 15, 321-353 (1986)).

Figure 4. mRNA expression patterns for the OPG 30 cDNA in human tissues. Northern blots were probed with a 32P-labeled rat cDNA insert (A, left two panels), or with the human cDNA insert (B, right panel).

Figure 5. Creation of transgenic mice expressing the OPG cDNA in hepatocytes. Northern blot expression 35 of HE-OPG transgene in mouse liver.

Figure 6. Increase in bone density in OPG transgenic mice. Panel A-F. Control Mice. G-J, OPG expressing mice. At necropsy, all animals were radiographed and photographs prepared. In A-F, the 5 radiographs of the control animals and the one transgenic non-expressor (#28) are shown. Note that the bones have a clearly defined cortex and a lucent central marrow cavity. In contrast, the OPG (G-J) animals have a poorly defined cortex and increased 10 density in the marrow zone.

Figure 7. Increase in trabecular bone in OPG transgenic mice. A-D. Representative photomicrographs of bones from control animals. In A and B, low (4X, 10X) power images of the femurs are shown (Masson 15 Trichrome stain). Stains for tartrate resistant acid phosphatase (TRAP) demonstrate osteoclasts (see arrows) both resorbing cartilage (C) and trabecular bone (D). Note the flattened appearance of osteoclasts on trabecular bone. E-H. Representative photomicrographs 20 of bones from OPG-expressing animals. In E and F, low (4X, 10X) power images of the femurs are shown (Masson Trichrome stain). The clear region is the growth plate cartilage, blue stained area is bone, and the red area is marrow. Note that in contrast to the controls, the 25 trabecular bone has not been resorbed resulting in the absence of the usual marrow cavity. Also, the resulting trabeculae have a variegated appearance with blue and clear areas. The clear areas are remnants of growth plate cartilage that have never been remodelled. Based 30 on TRAP stains, these animals do have osteoclasts (see arrows) at the growth plate (G), which may be reduced in number. However, the surfaces of the trabeculae away from the growth plate are virtually devoid of osteoclasts (H), a finding that stands in direct 35 contrast with the control animals (see D).

Figure 8. HE-OPG expressors do not have a defect in monocyte-macrophage development. One cause for osteopetrosis in mice is defective M-CSF production due to a point mutation in the M-CSF gene. This results in 5 a marked deficit of circulating and tissue based macrophages. The peripheral blood of OPG expressors contained monocytes as assessed by H1E analysis. To affirm the presence of tissue macrophages, immnohistochemistry was performed using F480 10 antibodies, which recognize a cell surface antigen on murine macrophages. A and C show low power (4X) photomicrographs of the spleens from normal and CR1 overexpressors. Note that both animals have numerous F480 positive cells. Monocyte-macrophages were also 15 present in the marrow of normal (B) and HE-OPG overexpressors (D) (40X).

Figure 9. Structure and sequence of mouse and human OPG cDNA clones. A, B. Mouse cDNA and protein sequence. C, D. Human cDNA and protein sequence. The 20 predicted signal peptides are underlined, and potential sites of N-linked glycosylation are indicated in bold. E, F. Sequence alignment and comparison of rat, mouse and human OPG amino acid sequences.

Figure 10. Comparison of conserved sequences in 25 extracellular domain of TNFR-I and human OPG. PrettyPlot (Wisconsin GCG Package, Version 8.1) of the TNFR1 and OPG alignment described in example 6. Top line, human TNFR1 sequences encoding domains 1-4. Bottom line, human OPG sequences encoding domains 1-4. 30 Conserved residues are highlighted by rectangular boxes.

Figure 11. Three-dimensional representation of 35 human OPG. Side-view of the Molescript display of the predicted 3-dimensional structure of human OPG residues 25 through 163, (wide line), co-crystallized with human

TNF $\beta$  (thin line). As a reference for orientation, the bold arrows along the OPG polypeptide backbone are pointing in the N-terminal to C-terminal direction. The location of individual cysteine residue side chains are 5 inserted along the polypeptide backbone to help demonstrate the separate cysteine-rich domains. The TNF $\beta$  molecule is aligned as described by Banner *et al.* (1993).

Figure 12. Structure of OPG cysteine-rich domains.  
10 Alignment of the human (top line SEQ ID NO:136) and mouse (bottom line) OPG amino acid sequences highlighting the predicted domain structure of OPG. The polypeptide is divided into two halves; the N-terminus (A), and C-terminus (B). The N-terminal half is  
15 predicted to contain four cysteine rich domains (labeled 1-4). The predicted intrachain disulfide bonds are indicated by bold lines, labeled "SS1", "SS2", or "SS3". Tyrosine 28 and histidine 75 (underlined) are predicted to form an ionic interaction. Those amino  
20 acids predicted to interact with an OPG ligand are indicated by bold dots above the appropriate residue. The cysteine residues located in the C-terminal half of OPG are indicated by rectangular boxes.

Figure 13. Expression and secretion of full length  
25 and truncated mouse OPG-Fc fusion proteins. A. Map indicating points of fusion to the human IgG1 Fc domain are indicated by arrowheads. B. Silver stain of a SDS-polyacrylamide gel of conditioned media obtained from cells expressing either Fl.Fc (Full length OPG fused to Fc at Leucine 401) or CT.Fc (Carboxy-terminal truncated OPG fused to Fc at threonine 180) fusion protein expression vectors. Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line. C. Western blot of  
30 conditioned media obtained from Fl.Fc and CT.Fc fusion  
35

protein expression vectors probed with anti-human IgG1 Fc domain (Pierce). Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line.

5       Figure 14. Expression of human OPG in E. coli. A. Construction of a bacterial expression vector. The LORF of the human OPG gene was amplified by PCR, then joined to a oligonucleotide linker fragment (top strand is SEQ ID NO:137; bottom strand is SEQ ID NO:127), and ligated  
10 into pAMG21 vector DNA. The resulting vector is capable of expressing OPG residues 32-401 linked to a N-terminal methionine residue. B SDS-PAGE analysis of uninduced and induced bacterial harboring the pAMG21-human OPG -32-401 plasmid. Lane 1, MW standards; lane  
15 2, uninduced bacteria; lane 3, 30°C induction; lane 4, 37°C induction; lane 5, whole cell lysate from 37°C induction; lane 6, soluble fraction of whole cell lysate; lane 7, insoluble fraction of whole cell lysate; lane 8, purified inclusion bodies obtained from  
20 whole cell lysate.

Figure 15. Analysis of recombinant murine OPG produced in CHO cells by SDS-PAGE and western blotting. An equal amount of CHO conditioned media was applied to each lane shown, and was prepared by treatment with  
25 either reducing sample buffer (left lane), or non-reducing sample buffer (right lane). After electrophoresis, the resolved proteins were transferred to a nylon membrane, then probed with anti-OPG antibodies. The relative positions of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.  
30

Figure 16. Pulse-chase analysis of recombinant murine OPG produced in CHO cells. CHO cells were pulse-labeled with  $^{35}\text{S}$ -methionine/cysteine, then chased  
35 for the indicated time. Metabolically labeled cultures were separated into both conditioned media and cells,

and detergent extracts were prepared from each, clarified, then immunoprecipitated with anti-OPG antibodies. The immunoprecipitates were resolved by SDS-PAGE, and exposed to film. Top left and right panels; samples analyzed under non-reducing conditions. Lower left and right panels; samples analyzed under reducing conditions. Top and bottom left panels; Cell extracts. Top and bottom right panels; Conditioned media extracts. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 17. Expression of OPG in the CTLL-2 cell line. Serum-free conditioned media from CTLL-2 cells and CHO-mu OPG [1-401] transfected cells was prepared, concentrated, then analyzed by non-reducing SDS-PAGE and western blotting. Left lane; CTLL-2 conditioned media. Right lane; CHO-muOPG conditioned media. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 18. Detection of OPG expression in serum samples and liver extracts obtained from control and OPG transgenic mice. Transgenic mice were constructed as described in Example 4. OPG expression was visualized after SDS-PAGE followed by Western blotting using anti-OPG antibodies.

Figure 19. Effects of huOPG [22-401]-Fc fusion protein on osteoclast formation in vitro. The osteoclast forming assay was performed as described in Example 11A in the absence (control) or presence of the indicated amounts of huOPG [22-401]-Fc fusion. Osteoclast formation was visualized by histochemical staining for tartrate acid phosphatase (TRAP). A. OPG added to 100 ng/ml. D. OPG added to 0.1 ng/ml. E. OPG added to 0.01 ng/ml. F. OPG added to 0.001 ng/ml. G. Control. No OPG added.

Figure 20. Decrease in osteoclast culture TRAP activity with increasing amounts of OPG. Indicated concentrations of huOPG [22-401]-Fc fusion protein were added to osteoclast forming assay and TRAP activity 5 quantitated as described in Example 11A.

Figure 21. Effect of OPG on a terminal stage of osteoclast differentiation. huOPG [22-401]-Fc fusion was added to the osteoclast forming assay during the intermediate stage of osteoclast maturation (days 5-6; 10 OPG-CTL) or during the terminal stage of osteoclast maturation (days 7-15; CTL-OPG). TRAP activity was quantitated and compared with the activity observed in the absence of OPG (CTL-CTL) in the presence of OPG throughout (OPG-OPG).

Figure 22. Effects of IL-1 $\beta$ , IL-1 $\alpha$  and OPG on blood ionized calcium in mice. Levels of blood ionized calcium were monitored after injection of IL-1 $\beta$  alone, IL-1 $\alpha$  alone, IL-1 $\beta$  plus muOPG [22-401]-Fc, IL-1 $\alpha$  plus MuOPG [22-401]-Fc, and muOPG [22-401]-Fc alone. Control 20 mice received injections of phosphate buffered saline (PBS) only. IL-1 $\beta$  experiment shown in A; IL-1 $\alpha$  experiment shown in B.

Figure 23. Effects of OPG on calvarial osteoclasts in control and IL-1-treated mice. Histological methods 25 for analyzing mice calvarial bone samples are described in Example 11B. Arrows indicate osteoclasts present in day 2-treated mice. Calvarial samples of mice receiving four PBS injections daily (A), one injection of IL-1 and three injections of PBS daily (B), one injection of PBS and three injections of OPG daily (C), one 30 injection of IL-1 and three injections of OPG daily.

Figure 24. Radiographic analysis of bone accumulation in marrow cavity of normal mice. Mice were injected subcutaneously with saline (A) or muOPG [22-

401]-Fc fusion (5mg/kg/d) for 14 days (B) and bone density determined as described in Example 11C.

Figure 25. Histomorphometric analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C.

Figure 26. Histology analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 10 11C. A. Saline injection B. Injection of muOPG [22-401]-Fc fusion.

Figure 27. Activity of OPG administered to ovariectomized rats. In this two week experiment the trend to reduced bone density appears to be blocked by 15 OPG or other anti-resorptive therapies. DEXA measurements were taken at time of ovariectomy and at week 1 and week 2 of treatment. The results are expressed as % change from the initial bone density (Mean +/- SEM).

20 Figure 28. Bone density in the femoral metaphysis, measured by histomorphometric methods, tends to be lower in ovariectomized rats (OVX) than sham operated animals (SHAM) 17 days following ovariectomy. This effect was blocked by OPG-Fc, with OPG-Fc treated 25 ovariectomized rats (OVX+OPG) having significantly higher bone density than vehicle treated ovariectomized rats (OVX). (Mean +/- SEM).

Figure 29A through 29G. Sequence of OPG-Fc. DNA and encoded protein sequences are shown. Restriction sites for various nucleases are noted above the DNA sequence.

30 Figures 30A through 30D. Effects of OPG-Fc during the course of adjuvant arthritis I male Lewis rats. Paws from rats with adjuvant arthritis induced by 0.5mg 35 mycobacteria in oil were analyzed by DEXA for bone mineral density (BMD). Evaluation of BMD, a 29mm X 25mm

box was centered at the calcaneus (expt AdA-14 2/99, Amgen nb#22957 p47-49). \* compared to normal, # compared to vehicle P<0.05 Mann-Whitney U test.

- Figures 31A and 31B. Combination treatment with  
5 OPG-Fc and sTNF-RI on Adjuvant Arthritis in Male Lewis  
Rats. Area under the curve (AUC) for measurement of paw  
swelling and BMD were measured as described above for  
Figure 33 and in the examples hereinafter.

Detailed Description of the InventionOPG proteins

The term "OPG protein" refers collectively to the novel member of the tumor necrosis factor receptor family described hereinafter, variants and truncations thereof that maintain OPG's activity in increasing bone density, and antibodies to OPG ligand that maintain OPG's activity in increasing bone density. An exemplary assay for measuring such activity is shown in figure 6 and the accompanying text. Exemplary OPG proteins are polypeptides comprising the consensus of the rat, mouse and human sequences (figure 9C), OPG-Fc fusions (figures 13, 29), or the rat, mouse or human OPG sequences (figures 2, 9).

OPG was identified as follows. A novel member of the tumor necrosis factor receptor (TNFR) superfamily was identified as an expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library . The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in Examples 1 and 6. The rat, mouse and human genes are shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), respectively. All three sequences showed strong similarity to the extracellular domains of TNFR family members. None of the full-length cDNA clones isolated encoded transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human gene spanning nucleotides 1200-1353 shown in Figure 9D was deposited in the Genebank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat, mRNA expression was detected in kidney, liver, placenta and

heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the same tissues along with lymph node, thymus, spleen and 5 appendix.

The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long 10 bones (femurs), vertebrae and flat bones (pelvis). Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and 15 resorption which has led to a marked accumulation of bone and cartilage. A decrease in the number of trabecular osteoclasts in the bones of OPG expressor animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent bone resorption, a process mediated by osteoclasts. In 20 view of the activity in transgenic expressors, the TNFR-related proteins described herein are termed OPGs.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse OPG in 293 cells and human OPG in E. coli is described 25 in Examples 7 and 8. Mouse OPG was produced as an Fc fusion which was purified by Protein A affinity chromatography. Also described in Example 7 is the expression of full-length and truncated human and mouse OPG polypeptides in CHO and 293 cells either as fusion 30 polypeptides to the Fc region of human IgG1 or as unfused polypeptides. The expression of full-length and truncated human and mouse OPGs in E. coli either as Fc fusion polypeptides or as unfused polypeptides is described in Example 8. Purification of recombinantly 35 produced mammalian and bacterial OPG is described in Example 10.

The biological activity of OPG was determined using an in vitro osteoclast maturation assay, an in vivo model of interleukin-1 (IL-1) induced hypercalcemia, and injection studies of bone density in 5 normal mice (see Example 11). The following OPG recombinant proteins produced in CHO or 293 cells demonstrated activity in the in E. coli osteoclast maturation assay: muOPG [22-185]-Fc, muOPG [22-194]-Fc, muOPG [22-401]Fc, muOPG [22-401], huOPG [22-201]-Fc, 10 huOPG [22-401]-Fc. muOPG [22-180]-Fc produced in CHO cells and huOPG met[32-401] produced in E. coli did not demonstrate activity in the in vitro assay.

OPG from several sources was produced as a dimer and to some extent as a higher multimer. Rat OPG [22- 15 401] produced in transgenic mice, muOPG [22-401] and huOPG [22-401] produced as a recombinant polypeptide in CHO cells, and OPG expressed as a naturally occurring product from a cytotoxic T cell line were predominantly dimers and trimers when analyzed on nonreducing SDS 20 gels (see Example 9). Truncated OPG polypeptides having deletions in the region of amino acids 186-401 (e.g., OPG [1-185] and OPG [1-194]) were predominantly monomeric suggesting that the region 186-401 may be involved in self-association of OPG polypeptides. 25 However, huOPG met[32-401] produced in E. coli was largely monomeric.

OPG may be important in regulating bone resorption. The protein appears to act as a soluble receptor of the TNF family and may prevent a receptor- 30 ligand interaction involved in the osteolytic pathway. One aspect of the regulation appears to be a reduction in the number of osteoclasts.

OPG proteins encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]- 35 Fc fusion, rat [1-180]-Fc fusion, mouse [1-401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180],

human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO:121 (rat), SEQ ID NO:123 (mouse) and SEQ ID NO:125 (human). Also  
5 encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of OPG; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of OPG, in  
10 particular deletion of the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain. In one embodiment, OPG has from 1 to about 216 amino acids  
15 deleted from the carboxy terminus. In another embodiment, OPG has from 1 to about 10 amino acids deleted from the mature amino terminus (wherein the mature amino terminus is at residue 22) and, optionally, has from 1 to about 216 amino acids deleted  
20 from the carboxy terminus.

Additional OPG proteins encompassed by the invention include the following: human [22-180]-Fc fusion, human [22-201]-Fc fusion, human [22-401]-Fc fusion, mouse [22-185]-Fc fusion, mouse [22-194]-Fc fusion. These polypeptides are produced in mammalian host cells, such as CHO or 293 cells, Additional OPG polypeptides encompassed by the invention which are expressed in prokaryotic host cells include the following: human met[22-401], Fc-human met[22-401] fusion (Fc region is fused at the amino terminus of the full-length OPG coding sequence as described in Example 8), human met[22-401]-Fc fusion (Fc region fused to the full-length OPG sequence), Fc-mouse met[22-401] fusion, mouse met[22-401]-Fc fusion, human met[27-401], human met[22-185], human met[22-189], human met[22-194], human met[22-194] (P25A), human met [22-194] (P26A),  
30  
35

human met[27-185], human met[27-189], human met[27-194], human met-arg-gly-ser-(his)<sub>6</sub> [22-401], human met-lys [22-401], human met-(lys)<sub>3</sub>-[22-401], human met[22-401]-Fc (P25A), human met[22-401] (P25A), human  
5 met[22-401] (P26A), human met[22-401] (P26D), mouse met[22-401], mouse met[27-401], mouse met[32-401], mouse met[27-180], mouse met[22-189], mouse met[22-194], mouse met[27-189], mouse met[27-194], mouse  
met-lys[22-401], mouse HEK[22-401] (A45T), mouse met-  
10 lys-(his)<sub>7</sub>[22-401], mouse met-lys[22-401]-(his)<sub>7</sub> and mouse met[27-401] (P33E, G36S, A45P). It is understood that the above OPG polypeptides produced in prokaryotic host cells have an amino-terminal methionine residue, if such a residue is not indicated. In specific  
15 examples, OPG-Fc fusion were produced using a 227 amino acid region of human IgG1- $\gamma$ 1 was used having the sequence as shown in Ellison *et al.* (1982) Nuc. Acids Res. 10: 4071-9. However, variants of the Fc region of human IgG may also be used.

20 Analysis of the biological activity of carboxy-terminal OPG truncations fused to the human IgG1 Fc region indicates a portion of OPG of about 164 amino acids which is required for activity. This region encompasses amino acids 22-185, preferably those in  
25 Figure 9C-9D (SEQ ID NO:125), and comprises four cysteine-rich domains characteristic of the cysteine-rich domains of TNFR extracellular domains. Proteins comprising this 164 amino acid sequence are within the meaning of "OPG protein" in this invention.

30 OPG proteins of the invention also may be isolated and purified from other polypeptides present in tissues, cell lines and transformed host cells expressing OPG, or purified from components in cell cultures containing the secreted protein. In one embodiment, the polypeptide is free from association  
35

with other human proteins, such as the expression product of a bacterial host cell.

A method for the purification of OPG from natural sources and from transfected host cells is also included. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG antibody or biotin-streptavidin affinity complex and the like.

#### IL-1 inhibitors

One of the most potent inflammatory cytokines yet discovered is interleukin-1 (IL-1). IL-1 is thought to be a key mediator in many diseases and medical conditions. It is manufactured (though not exclusively) by cells of the macrophage/monocyte lineage and may be produced in two forms: IL-1 alpha (IL-1 $\alpha$ ) and IL-1 beta (IL-1 $\beta$ ).

A disease or medical condition is considered to be an "interleukin-1 mediated disease" if the spontaneous or experimental disease or medical condition is associated with elevated levels of IL-1 in bodily fluids or tissue or if cells or tissues taken from the body produce elevated levels of IL-1 in culture. In many cases, such interleukin-1 mediated diseases are also recognized by the following additional two conditions: (1) pathological findings associated with the disease or medical condition can be mimicked experimentally in animals by administration of IL-1 or upregulation of expression of IL-1; and (2) a pathology induced in experimental animal models of the disease or medical condition can be inhibited or abolished by treatment with agents that inhibit the action of IL-1. In most interleukin-1 mediated diseases at least two of

the three conditions are met, and in many interleukin-1 mediated diseases all three conditions are met.

A non-exclusive list of acute and chronic interleukin-1 (IL-1)-mediated diseases includes but is  
5 not limited to the following:

acute pancreatitis;  
ALS;  
Alzheimer's disease;  
cachexia/anorexia, including AIDS-induced  
10 cachexia;  
asthma and other pulmonary diseases;  
atherosclerosis;  
autoimmune vasculitis;  
chronic fatigue syndrome;  
15 Clostridium associated illnesses, including  
Clostridium-associated diarrhea;  
coronary conditions and indications,  
including congestive heart failure, coronary  
restenosis, myocardial infarction, myocardial  
20 dysfunction (e.g., related to sepsis), and coronary  
artery bypass graft;  
cancer, such as multiple myeloma and  
myelogenous (e.g., AML and CML) and other leukemias, as  
well as tumor metastasis;  
25 diabetes (e.g., insulin diabetes);  
endometriosis;  
fever;  
fibromyalgia;  
glomerulonephritis;  
30 graft versus host disease/transplant  
rejection;  
hemorrhagic shock;  
hyperalgesia;  
inflammatory bowel disease;

inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis;

- 5 inflammatory eye disease, as may be associated with, for example, corneal transplant; ischemia, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration);
- 10 Kawasaki's disease; learning impairment; lung diseases (e.g., ARDS); multiple sclerosis; myopathies (e.g., muscle protein metabolism, esp. in sepsis); neurotoxicity (e.g., as induced by HIV); osteoporosis; pain, including cancer-related pain; Parkinson's disease;
- 20 periodontal disease; pre-term labor; psoriasis; reperfusion injury; septic shock;
- 25 side effects from radiation therapy; temporal mandibular joint disease; sleep disturbance; uveitis; or an inflammatory condition resulting from
- 30 strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes.

Interleukin-1 inhibitors may be from any protein capable of specifically preventing activation of cellular receptors to IL-1, which may result from any 35 number of mechanisms. Such mechanisms include downregulating IL-1 production, binding free IL-1,

interfering with IL-1 binding to its receptor,  
interfering with formation of the IL-1 receptor complex  
(i.e., association of IL-1 receptor with IL-1 receptor  
accessory protein), or interfering with modulation of  
5 IL-1 signaling after binding to its receptor. Classes  
of interleukin-1 inhibitors include:  
interleukin-1 receptor antagonists such as IL-1ra,  
as described below;  
anti-IL-1 receptor monoclonal antibodies (e.g., EP  
10 623674), the disclosure of which is hereby incorporated  
by reference;  
IL-1 binding proteins such as soluble IL-1  
receptors (e.g., U. S. Pat. No. 5,492,888, U. S. Pat.  
No. 5,488,032, and U. S. Pat. No. 5,464,937, U. S. Pat.  
15 No. 5,319,071, and U.S. Pat. No. 5,180,812, the  
disclosures of which are hereby incorporated by  
reference);  
anti-IL-1 monoclonal antibodies (e.g., WO 9501997,  
WO 9402627, WO 9006371, U.S.Pat. No. 4,935,343, EP  
20 364778, EP 267611 and EP 220063, the disclosures of  
which are hereby incorporated by reference);  
IL-1 receptor accessory proteins and antibodies  
thereto (e.g., WO 96/23067 and WO 99/37773, the  
disclosure of which is hereby incorporated by  
25 reference);  
inhibitors of interleukin-1 beta converting enzyme  
(ICE) or caspase I (e.g., WO 99/46248, WO 99/47545, and  
WO 99/47154, the disclosures of which are hereby  
incorporated by reference), which can be used to  
30 inhibit IL-1 beta production and secretion;  
interleukin-1beta protease inhibitors;  
and other compounds and proteins which block *in  
vivo* synthesis or extracellular release of IL-1.  
Exemplary IL-1 inhibitors are disclosed in the  
35 following references:

US Pat. Nos. 5,747,444; 5,359,032; 5,608,035;  
5,843,905; 5,359,032; 5,866,576; 5,869,660; 5,869,315;  
5,872,095; 5,955,480; 5,965,564;

International (WO) patent applications 98/21957,  
5 96/09323, 91/17184, 96/40907, 98/32733, 98/42325,  
98/44940, 98/47892, 98/56377, 99/03837, 99/06426,  
99/06042, 91/17249, 98/32733, 98/17661, 97/08174,  
95/34326, 99/36426, 99/36415.

European (EP) patent applications 534978 and  
10 894795.

French patent application FR 2762514.

The disclosures of all of the aforementioned references  
are hereby incorporated by reference.

For purposes of the present invention, IL-1ra and  
15 variants and derivatives thereof as discussed  
hereinafter are collectively termed "IL-1ra  
protein(s)". The molecules described in the above  
references and the variants and derivatives thereof  
discussed hereinafter are collectively termed "IL-1  
20 inhibitors."

Interleukin-1 receptor antagonist (IL-1ra) is a  
human protein that acts as a natural inhibitor of  
interleukin-1 and which is a member of the IL-1 family  
member which includes IL-1 $\alpha$  and IL-1 $\beta$ . Preferred  
25 receptor antagonists (including IL-1ra and variants and  
derivatives thereof), as well as methods of making and  
using thereof, are described in U.S. Patent No.  
5,075,222; WO 91/08285; WO 91/17184; AU 9173636;  
WO 92/16221; WO93/21946; WO 94/06457; WO 94/21275;  
30 FR 2706772; WO 94/21235; DE 4219626, WO 94/20517; WO  
96/22793; WO 97/28828; and WO 99/36541, the disclosures  
of which are incorporated herein by reference. The  
proteins include glycosylated as well as non-  
glycosylated IL-1 receptor antagonists.

Specifically, three useful forms of IL-1ra and variants thereof are disclosed and described in the 5,075,222 patent. The first of these, called "IL-1i" in the '222 patent, is characterized as a 22-23 kD molecule on SDS-PAGE with an approximate isoelectric point of 4.8, eluting from a Mono Q FPLC column at around 52 mM NaCl in Tris buffer, pH 7.6. The second, IL-1ra $\beta$ , is characterized as a 22-23 kD protein, eluting from a Mono Q column at 48 mM NaCl. Both IL-1ra $\alpha$  and IL-1ra $\beta$  are glycosylated. The third, IL-1rax, is characterized as a 20 kD protein, eluting from a Mono Q column at 48 mM NaCl, and is non-glycosylated. 5,075,222 patent also discloses methods for isolating the genes responsible for coding the inhibitors, cloning the gene in suitable vectors and cell types, and expressing the gene to produce the inhibitors.

Those skilled in the art understand that many combinations of deletions, insertions and substitutions (individually or collectively "variant(s)") can be made within the amino acid sequences of IL-1ra, provided that the resulting molecule is biologically active (e.g., possesses the ability to inhibit IL-1). See "Variants of Proteins" hereinafter.

#### TNF- $\alpha$ inhibitors

Many diseases and medical conditions are mediated by TNF and are usually categorized as inflammatory conditions. A "TNF-mediated disease" is a spontaneous or experimental disease or medical condition is associated with elevated levels of TNF in bodily fluids or tissue or if cells or tissues taken from the body produce elevated levels of TNF in culture. In many cases, such TNF-mediated diseases may also be recognized by (1) pathological findings associated with the disease or medical condition can be mimicked experimentally in animals by the administration or

upregulation of expression of TNF or (2) a pathology induced in experimental animal models of the disease or medical condition can be inhibited or abolished by treatment with agents that inhibit the action of TNF. A 5 non-exclusive list of acute and chronic TNF-mediated diseases includes but is not limited to the following:

cachexia/anorexia;

cancer (e.g., leukemias);

chronic fatigue syndrome;

10 coronary conditions and indications, including congestive heart failure, coronary restenosis, myocardial infarction, myocardial dysfunction (e.g., related to sepsis), and coronary artery bypass graft;

depression;

15 diabetes, including juvenile onset Type 1, diabetes mellitus, and insulin resistance (e.g., as associated with obesity);

endometriosis, endometritis, and related conditions;

20 fibromyalgia or analgesia;

graft versus host rejection;

hyperalgesia;

inflammatory bowel diseases, including Crohn's disease and Clostridium difficile-associated diarrhea;

25 ischemia, including cerebral ischemia (brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration);

lung diseases (e.g., adult respiratory distress syndrome, asthma, and pulmonary fibrosis);

30 multiple sclerosis;

neuroinflammatory diseases;

ocular diseases and conditions, including corneal transplant, ocular degeneration and uveitis;

pain, including cancer-related pain;

35 pancreatitis;

periodontal diseases;

Pityriasis rubra pilaris (PRP);  
prostatitis (bacterial or non-bacterial) and  
related conditions;  
psoriasis and related conditions;  
5 pulmonary fibrosis;  
reperfusion injury;  
rheumatic diseases, including rheumatoid  
arthritis, osteoarthritis, juvenile (rheumatoid)  
arthritis, seronegative polyarthritis, ankylosing  
10 spondylitis, Reiter's syndrome and reactive arthritis,  
Still's disease, psoriatic arthritis, enteropathic  
arthritis, polymyositis, dermatomyositis, scleroderma,  
systemic sclerosis, vasculitis (e.g., Kawasaki's  
disease), cerebral vasculitis, Lyme disease,  
15 staphylococcal-induced ("septic") arthritis, Sjögren's  
syndrome, rheumatic fever, polychondritis and  
polymyalgia rheumatica and giant cell arteritis);  
septic shock;  
side effects from radiation therapy;  
20 systemic lupus erythematosus (SLE);  
temporal mandibular joint disease;  
thyroiditis;  
tissue transplantation or an inflammatory  
condition resulting from strain, sprain, cartilage  
25 damage, trauma, orthopedic surgery, infection (e.g.,  
HIV, Clostridium difficile and related species) or  
other disease process.

TNF- $\alpha$  inhibitors may act by downregulating or  
inhibiting TNF production, binding free TNF,  
30 interfering with TNF binding to its receptor, or  
interfering with modulation of TNF signaling after  
binding to its receptor. The term "TNF- $\alpha$  inhibitor"  
thus includes solubilized TNF receptors, antibodies to  
TNF, antibodies to TNF receptor, inhibitors of TNF- $\alpha$

converting enzyme (TACE), and other molecules that affect TNF activity.

TNF- $\alpha$  inhibitors of various kinds are disclosed in the art, including the following references:

- 5 European patent applications 308 378; 422 339; 393  
438; 398 327; 412 486; 418 014, 417 563, 433 900; 464  
533; 512 528; 526 905; 568 928; EP 607 776 (use of  
leflunomide for inhibition of TNF- $\alpha$ ); 663 210; 542 795;  
818 439; 664 128; 542 795; 741 707; 874 819 ; 882 714;  
10 880 970; 648 783; 731 791; 895 988; 550 376; 882 714;  
853 083; 550 376; 943 616; 939 121; 614 984 ; 853 083  
U.S. Patent Nos. 5,136,021; 5,929,117; 5,948,638;  
5,807,862; 5,695,953; 5,834,435; 5,817,822; 5830742;  
5,834,435; 5,851,556; 5,853,977; 5,359,037; 5,512,544;  
15 5,695,953; 5,811,261; 5,633,145; 5,863,926; 5,866,616;  
5,641,673; 5,869,677; 5,869,511; 5,872,146; 5,854,003;  
5,856,161; 5,877,222; 5,877,200; 5,877,151; 5,886,010;  
5,869,660; 5,859,207; 5,891,883; 5,877,180; 5,955,480;  
5,955,476; 5,955,435; 5,994,351; 5,990,119; 5,952,320;  
20 5,962,481;  
International (WO) patent applications 90/13575,  
91/03553, 92/01002, 92/13095, 92/16221, 93/07863,  
93/21946, 93/19777, 95/34326, 96/28546, 98/27298,  
98/30541, 96/38150, 96/38150, 97/18207, 97/15561,  
25 97/12902, 96/25861, 96/12735, 96/11209, 98/39326,  
98/39316, 98/38859, 98/39315, 98/42659, 98/39329,  
98/43959, 98/45268, 98/47863, 96/33172, 96/20926,  
97/37974, 97/37973, 97/47599, 96/35711, 98/51665,  
98/43946, 95/04045, 98/56377, 97/12244, 99/00364,  
30 99/00363, 98/57936, 99/01449, 99/01139, 98/56788,  
98/56756, 98/53842, 98/52948, 98/52937, 99/02510,  
97/43250, 99/06410, 99/06042, 99/09022, 99/08688,  
99/07679, 99/09965, 99/07704, 99/06041, 99/37818,  
99/37625, 97/11668, 99/50238, 99/47672, 99/48491;

Japanese (JP) patent applications 10147531, 10231285, 10259140, and 10130149, 10316570, 11001481, and 127,800/1991;

5 German (DE) application 19731521;  
British (GB) applications 2 218 101, 2 326 881, 2  
246 569.

The disclosures of all of the aforementioned references are hereby incorporated by reference.

For purposes of this invention, the molecules  
10 disclosed in these references and the sTNFRs and variants and derivatives of the sTNFRs and the molecules disclosed in the references (see below) are collectively termed "TNF- $\alpha$  inhibitors."

For example, EP 393 438 and EP 422 339 teach the  
15 amino acid and nucleic acid sequences of a soluble TNF receptor type I (also known as sTNFR-I or 30kDa TNF inhibitor) and a soluble TNF receptor type II (also known as sTNFR-II or 40kDa TNF inhibitor), collectively termed "sTNFRs", as well as modified forms thereof  
20 (e.g., fragments, functional derivatives and variants). EP 393 438 and EP 422 339 also disclose methods for isolating the genes responsible for coding the inhibitors, cloning the gene in suitable vectors and cell types, and expressing the gene to produce the  
25 inhibitors.

sTNFR-I and sTNFR-II are members of the nerve growth factor/TNF receptor superfamily of receptors which includes the nerve growth factor receptor (NGF), the B cell antigen CD40, 4-1BB, the rat T-cell antigen 30 MRC OX40, the fas antigen, and the CD27 and CD30 antigens (Smith *et al.* (1990), *Science*, 248:1019-1023). The most conserved feature amongst this group of cell surface receptors is the cysteine-rich extracellular ligand binding domain, which can be divided into four  
35 repeating motifs of about forty amino acids and which

contains 4-6 cysteine residues at positions which are well conserved (Smith *et al.* (1990), *supra*).

EP 393 438 teaches a 40kDa TNF inhibitor Δ51 and a 40kDa TNF inhibitor Δ53, which are truncated versions 5 of the full-length recombinant 40kDa TNF inhibitor protein wherein 51 or 53 amino acid residues, respectively, at the carboxyl terminus of the mature protein are removed.

PCT Application No. PCT/US97/12244 teaches 10 truncated forms of sTNFR-I and sTNFR-II which do not contain the fourth domain (amino acid residues Thr<sup>127</sup>-Asn<sup>161</sup> of sTNFR-I and amino acid residues Pro<sup>141</sup>-Thr<sup>179</sup> of sTNFR-II); a portion of the third domain (amino acid residues Asn<sup>111</sup>-Cys<sup>126</sup> of sTNFR-I and amino acid 15 residues Pro<sup>123</sup>-Lys<sup>140</sup> of sTNFR-II); and, optionally, which do not contain a portion of the first domain (amino acid residues Asp<sup>1</sup>-Cys<sup>19</sup> of sTNFR-I and amino acid residues Leu<sup>1</sup>-Cys<sup>32</sup> of sTNFR-II). The truncated sTNFRs of the present invention include the proteins 20 represented by the formula R<sub>1</sub>-[Cys<sup>19</sup>-Cys<sup>103</sup>]-R<sub>2</sub> and R<sub>4</sub>-[Cys<sup>32</sup>-Cys<sup>115</sup>]-R<sub>5</sub>. These proteins are truncated forms of sTNFR-I and sTNFR-II, respectively.

By "R<sub>1</sub>-[Cys<sup>19</sup>-Cys<sup>103</sup>]-R<sub>2</sub>" is meant one or more proteins wherein [Cys<sup>19</sup>-Cys<sup>103</sup>] represents residues 19 through 103 of sTNFR-I, the amino acid residue numbering scheme of which is provided in Figure 1 to facilitate the comparison; wherein R<sub>1</sub> represents a methionylated or nonmethionylated amine group of Cys<sup>19</sup> or of amino-terminus amino acid residue(s) selected 25 from any one of Cys<sup>18</sup> to Asp<sup>1</sup> and wherein R<sub>2</sub> represents a carboxy group of Cys<sup>103</sup> or of carboxy-terminal amino acid residues selected from any one of Phe<sup>104</sup> to Leu<sup>110</sup>.

Exemplary truncated sTNFR-I of the present invention include the following molecules (collectively

termed 2.6D sTNFR-I): NH<sub>2</sub>-[Asp<sup>1</sup>-Cys<sup>105</sup>]-COOH (also referred to as sTNFR-I 2.6D/C105); NH<sub>2</sub>-[Asp<sup>1</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.6D/C106); NH<sub>2</sub>-[Asp<sup>1</sup>-Asn<sup>105</sup>]-COOH (also referred to as sTNFR-I 5 2.6D/N105); NH<sub>2</sub>-[Tyr<sup>9</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.3D/d8); NH<sub>2</sub>-[Cys<sup>19</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.3D/d18); and NH<sub>2</sub>-[Ser<sup>16</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.3D/d15), either methionylated or nonmethionylated, and variants and 10 derivatives thereof.

By "R<sub>3</sub>-[Cys<sup>32</sup>-Cys<sup>115</sup>]-R<sub>4</sub>" is meant one or more proteins wherein [Cys<sup>32</sup>-Cys<sup>115</sup>] represents residues Cys<sup>32</sup> through Cys<sup>115</sup> of sTNFR-II, the amino acid residue 15 numbering scheme of which is provided in Figure 2 to facilitate the comparison; wherein R<sub>3</sub> represents a methionylated or nonmethionylated amine group of Cys<sup>32</sup> or of amino-terminus amino acid residue(s) selected from any one of Cys<sup>31</sup> to Leu<sup>1</sup> and wherein R<sub>4</sub> represents a carboxy group of Cys<sup>115</sup> or of carboxy-terminal amino 20 acid residue(s) selected from any one of Ala<sup>116</sup> to Arg<sup>122</sup>.

Serine Protease Inhibitors

Endogenous proteolytic enzymes degrade invading organisms, antigen-antibody complexes, and certain tissue proteins that are no longer necessary or useful.

5 Infective agents may introduce additional proteolytic enzymes into the organism. Protease inhibitors regulate both endogenous and invading proteolytic enzymes.

A large number of naturally occurring protease inhibitors serve to control the endogenous proteases by limiting their reactions locally and temporally. In addition, the protease inhibitors may inhibit proteases introduced into the body by infective agents. Tissues that are particularly prone to proteolytic attack and infection, e.g. those of the respiratory tract, are

15 rich in protease inhibitors.

Protease inhibitors comprise approximately 10% of the human plasma proteins. At least eight inhibitors have been isolated from this source and characterized in the literature. These include alpha 2-macroglobulin

20 (alpha 2M), alpha 1-protease inhibitor (alpha 1PI), alpha 1-antichymotrypsin (alpha 1Achy), alpha 1-anticollagenase (alpha 1AC), and inter-alpha-trypsin inhibitor (I-alpha-I).

A disturbance of the protease/protease inhibitor balance can lead to protease-mediated tissue destruction, including emphysema, arthritis, glomerulonephritis, periodontitis, muscular dystrophy, tumor invasion and various other pathological conditions. In certain situations, e.g. severe

25 pathological processes such as sepsis or acute leukemia, the amount of free proteolytic enzymes present increases due to the release of enzyme from the secretory cells. In addition, or separately in other situations, a diminished regulating inhibitor capacity

of the organism may also cause alterations in the protease/protease inhibitor balance. An example of such a diminished regulating inhibitor capacity is an alpha 1-protease inhibitor deficiency, which is highly  
5 correlated with the development of pulmonary emphysema.

In organisms where such aberrant conditions are present, serious damage to the organism can occur unless measures can be taken to control the proteolytic enzymes. Therefore, protease inhibitors have been  
10 sought which are capable of being administered to an organism to control the proteolytic enzymes.

One protease that is of particular pharmacological interest is leukocyte elastase. Leukocyte elastase, when released extracellularly, degrades connective  
15 tissue and other valuable proteins. While it is necessary for a normally functioning organism to degrade a certain amount of connective tissue and other proteins, the presence of an excessive amount of leukocyte elastase has been associated with various  
20 pathological states, such as emphysema and rheumatoid arthritis. To counteract the effects of leukocyte elastase when it is present in amounts greater than normal, a protease inhibitor has been sought which is specific for leukocyte elastase. Such a protease  
25 inhibitor would be especially useful if it were capable of being isolated or prepared in a purified form and in sufficient quantities to be pharmaceutically useful

In the past, at least two leukocyte elastase inhibitors have been identified in the literature. One  
30 protein, described in Schiessler et al., "Acid-Stable Inhibitors of Granulocyte Neutral Proteases in Human Mucous Secretions: Biochemistry and Possible Biological Function", in Neutral Proteases of Human

Polymorphonuclear Leucocytes, Havemann *et al.* (eds), Urban and Schwarzenberg, Inc. (1978), was isolated from human seminal plasma and sputum and was characterized as being approximately 11 Kda in size with tyrosine as the N-terminal amino acid. The literature reports of this protein have only furnished a partial amino acid sequence, but even this partial sequence indicates that this protein varies substantially from the proteins of the present invention. The reports of the sequence of this protein, in combination with the complete amino acid sequence data for proteins of the present invention, indicate to the present inventors that the product sequenced by Schiessler *et al.* may have been a degraded protein which was not a single-polypeptide chain.

A second protein, isolated in one instance from human plasma, has been named alpha 1-protease inhibitor. Work on this protein has been summarized in a review by Travis and Salvesen, Ann. Rev. Biochem. 52: 20 655-709 (1983). The reports of the amino acid sequence of this protein indicate that it too differs substantially from the proteins of the present invention.

Trypsin is another protease of particular interest from a pharmacological standpoint. Trypsin is known to initiate degradation of certain soft organ tissue, such as pancreatic tissue, during a variety of acute conditions, such as pancreatitis. A variety of efforts have been directed toward the treatment of these conditions, without marked success, through the use of proteins which it was hoped would inhibit the action of trypsin. Illustrative of such efforts are attempts to use exogenous bovine trypsin inhibitors in treatment of

human pancreatitis. While such techniques have been attempted in Europe, they have not been approved as effective by the U.S. Food and Drug Administration.

Thus, there is a need for a protease inhibitor  
5 effective in neutralizing excess trypsin in a variety  
of acute and chronic conditions. As was the case with  
the leukocyte elastase inhibitor discussed above, a  
trypsin inhibitor would be particularly useful if it  
could be isolated and prepared in a purified form and  
10 in sufficient quantities to be pharmaceutically useful.

Cathepsin G is another protease present in large  
quantities in leukocytes. Cathepsin G is known to be  
capable of degrading in vitro a variety of valuable  
proteins, including those of the complement pathway  
15 Pancreatic elastase is another protease which may have  
a role in pancreatitis. Thus, inhibitors for these  
proteases are also of pharmaceutical value.

Leukocyte elastase, trypsin, cathepsin G and  
pancreatic elastase are examples of a class of  
20 proteases known as serine proteases, which have  
elements of common structure and mechanism. Their  
activity against different substrates and their  
sensitivity to different inhibitors are believed to  
result from changes in only a few amino acid residues.  
25 By analogy, it is possible to conceive of a class of  
serine protease inhibitors, also having common elements  
of structure and mechanism, in which changes in a  
relatively few amino acids will result in inhibition of  
different proteases, and that at least one member of  
30 this class will inhibit every serine protease of the  
former class.

A particularly preferred serine protease inhibitor  
is secretory leukocyte protease inhibitor (SLPI) and

fragments and analogues thereof. Also preferred are anti-leukoprotease (ALP), mucous protease inhibitor (MPI), human seminal plasma inhibitor-I (HUSI-I), bronchial mucus inhibitor (BMI), cervical mucus inhibitor (CUSI). These molecules are especially well-suited for use in conditions leading to bone loss because they are preferentially directed to the cartilage. Exemplary serine protease inhibitors are described in the following, each of which is hereby incorporated by reference: U. S. Pat. No. 4,760,130, issued July 26, 1988; U. S. Pat. No. 5,900,400, issued May 4, 1999, which discloses preferred SLPI analogues; and U. S. Pat. No. 5,633,227, issued May 27, 1997, which discloses preferred SLPI fragments. The molecules disclosed in the foregoing references as well as any variants or analogues thereof as described hereinafter are collectively termed "serine protease inhibitors."

IL-18 Inhibitors

IL-18 is a pro-inflammatory cytokine of somewhat recent discovery. IL-18 was found to induce interferon- $\gamma$  and was originally named interferon gamma inducing factor (IGIF). IL-1 upregulates IL-18 production, and IL-18 induces production of a number of proinflammatory cytokines, including IL-6 and MMP-1. Dinarello *et al.* (1998), J. Leukocyte Biol. 63: 658-64. Caspase I is also critical for IL-18 production. The art also suggested that TNF- $\alpha$  regulates IL-18 production, and it was found that simultaneous inhibition of TNF- $\alpha$  and IL-18 protected against liver toxicity. Faggioni *et al.* (2000), PNAS 97: 2367-72.

IL-18 acts *in vivo* through a receptor system reminiscent of the IL-1 system. IL-18 interacts with a cell surface receptor (IL-18R), which interacts with an accessory protein (IL-18RAcP). IL-18-mediated signaling proceeds upon formation of the complex of IL-18, IL-

18R, and IL-18RAcP. A natural inhibitor for IL-18 is IL-18bp. Although it bears insignificant sequence homology with IL-18R, IL-18bp's act as a "decoy receptors" by binding to IL-18 molecules and preventing 5 interaction with IL-18 and subsequent IL-18-mediated signaling.

The present invention concerns methods of treatment using IL-18 inhibitors in combination with the other classes of molecules described herein. Such 10 combination therapy is useful for treating inflammation and autoimmune diseases generally, as well as IL-1 mediated diseases and TNF-mediated diseases as defined hereinabove. In particular, combination therapy using IL-18 inhibitors is useful for treating arthritis 15 (particularly rheumatoid arthritis), systemic lupus erythematosus (SLE), graft versus host disease (GvHD), hepatitis and sepsis.

A number of classes of IL-18 inhibitors are known in the art, and all are useful in the present 20 invention. Suitable IL-18 inhibitors include antibodies binding to IL-18; antibodies binding to IL-18R; antibodies binding to IL-18RAcP; IL-18bp; IL-18R fragments (e.g., a solubilized extracellular domain of the IL-18 receptor), peptides binding to IL-18 and 25 preventing its interaction with IL-18R; peptides binding to IL-18R and preventing its interaction with IL-18 or with IL-18RAcP; peptides binding to IL-18RAcP and preventing its interaction with IL-18R; and small molecules preventing IL-18 production or interaction 30 between any of IL-18, IL-18R, and IL-18RAcP. Any of the foregoing, with the exception of small molecules, may be linked to half-life extending vehicles known in the art. Such vehicles include the Fc domain, polyethylene glycol, and dextran. These vehicles are reviewed in a 35 patent application entitled, "Modified Peptides as Therapeutic Agents," U.S. Ser. No. 09/428,082, PCT

appl. no. WO 99/25044, which is hereby incorporated by reference in its entirety.

Useful IL-18 inhibitors are described in the following references, which are hereby incorporated by reference: US Pat. No. 5,912,324, issued July 14, 1994; EP 0 962 531, published Dec. 8, 1999; EP 712 931, published Nov. 15, 1994; US Pat. No. 5,914,253, issued July 14, 1994; WO 97/24441, published July 10, 1997; US Pat. No. 6,060,283, issued May 9, 2000; EP 850 952, published Dec. 26, 1996; EP 864 585, published Sep. 16, 1998; WO 98/41232, published Sep. 24, 1998; US Pat. No. 6,054,487, issued April 25, 2000; WO 99/09063, published Aug 14, 1997; WO 99/22760, published Nov. 3, 1997; WO 99/37772, published Jan. 23, 1998; WO 15 99/37773, published March 20, 1998; EP 0 974 600, published Jan. 26, 2000; WO 00/12555, published Mar. 9, 2000; Japanese patent application JP 111,399/94, published Oct. 31, 1997; Israel patent application IL 121554 A0, published Feb. 8, 1998.

20 Variants of proteins

Those skilled in the art will understand that one may make many molecules derived in sequence from the aforementioned molecules in which amino acids have been deleted ("deletion variants"), inserted ("addition variants"), or substituted ("substitution variants"). Molecules having such substitutions, additions, deletions, or any combination thereof are termed individually or collectively "variant(s)"). Such variants should, however, maintain at some level (including a reduced level) the relevant activity of the unmodified or "parent" molecule (e.g., an STNFR variant possesses the ability to bind TNF). Hereinafter, "parent molecule" refers to an unmodified molecule or a variant molecule lacking the particular variation under discussion; for example, when

discussing substitution below, the parent molecule may be a deletion variant.

Variants may be rapidly screened to assess their physical properties. It will be appreciated that such 5 variant(s) will demonstrate similar properties to the unmodified molecule, but not necessarily all of the same properties and not necessarily to the same degree as the corresponding parent molecule.

There are two principal variables in the 10 construction of amino acid sequence variant(s): the location of the mutation site and the nature of the mutation. In designing variant(s), the location of each mutation site and the nature of each mutation will depend on the biochemical characteristic(s) to be 15 modified. Each mutation site can be modified individually or in series, e.g., by (1) deleting the target amino acid residue, (2) inserting one or more amino acid residues adjacent to the located site or (3) substituting first with conservative amino acid 20 choices and, depending upon the results achieved, then with more radical selections.

Amino acid sequence deletions generally range from about 1 to 30 amino acid residues, preferably from about 1 to 20 amino acid residues, more preferably from 25 about 1 to 10 amino acid residues and most preferably from about 1 to 5 contiguous residues. Amino-terminal, carboxy-terminal and internal intrasequence deletions are contemplated. Deletions within the amino acid sequences of OPG or the sTNFRs may be made, for 30 example, in regions of low homology with the sequences of other members of the NGF/TNF receptor family. In the case of IL-1ra, deletions may be made in regions of low homology in the IL-1 family (which comprises IL-1  $\alpha$ , IL-1  $\beta$ , and IL-1ra). Deletions in areas of substantial 35 homology with other members of the family will be more

likely to significantly modify the biological activity. Specifically, the sequence similarity among NGF/TNF receptor family members is particularly high in the region corresponding to the first two disulfide loops of domain 1, the whole of domain 2, and the first disulfide loop of domain 3 (Banner *et al.* (1993), *Cell*, 73:431-445). The number of total deletions and/or consecutive deletions preferably will be selected so as to preserve the tertiary structure in the affected domain, e.g., cysteine crosslinking.

An amino acid sequence addition may include insertions of an amino- and/or carboxyl-terminal fusion ranging in length from one residue to one hundred or more residues, as well as internal intrasequence insertions of single or multiple amino acid residues. Internal additions may range generally from about 1 to 20 amino acid residues, preferably from about 1 to 10 amino acid residues, more preferably from about 1 to 5 amino acid residues, and most preferably from about 1 to 3 amino acid residues. Additions within the amino acid sequences of OPG or the sTNFRs may be made in regions of low homology with the sequences of other members of the NGF/TNF receptor family. Additions within the amino acid sequence of OPG or the sTNFRs in areas of substantial homology with the sequences of other members of the NGF/TNF receptor family will be more likely to significantly modify the biological activity. Additions preferably include amino acid sequences derived from the sequences of the NGF/TNF receptor family members.

An amino-terminus addition is contemplated to include the addition of a methionine (for example, as an artifact of the direct expression in bacterial recombinant cell culture). A further example of an amino-terminal addition includes the fusion of a signal sequence to the amino-terminus of a mature molecule in

order to facilitate its secretion from recombinant host cells. Such signal sequences generally will be obtained from and thus be homologous to the intended host cell species. For prokaryotic host cells that do not

5 recognize and process the native signal sequence of the mature molecule, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase or heat-stable enterotoxin II leader

10 sequences. For expression in yeast cells the signal sequence may be selected, for example, from the group of the yeast invertase, alpha factor or acid phosphatase leader sequences. For mammalian cell expression, the native signal sequences (see, e.g., EP

15 393 438 and EP 422 339 for sTNFRs) are satisfactory, although other mammalian signal sequences may be suitable; for example, sequences derived from other NGF/TNF receptor family members.

An example of an amino- or a carboxy-terminus

20 addition includes chimeric proteins comprising the amino-terminal or carboxy-terminal fusion of the parent molecules with all or part of the constant domain of the heavy or light chain of human immunoglobulin (individually or collectively, ("Fc variant(s)"). Such

25 chimeric polypeptides are preferred wherein the immunoglobulin portion of each comprises all of the domains except the first domain of the constant region of the heavy chain of human immunoglobulin such as IgG (e.g., IgG1 or IgG3), IgA, IgM or IgE. A skilled

30 artisan will appreciate that any amino acid of the immunoglobulin portion can be deleted or substituted with one or more amino acids, or one or more amino acids can be added as long as the parent molecule still maintains some level of its relevant activity and the

35 immunoglobulin portion shows one or more of its characteristic properties.

Another group of variant(s) is amino acid substitution variant(s). These are variant(s) wherein at least one amino acid residue in a parent molecule is removed and a different residue inserted in its place.

- 5 Substitution variant(s) include allelic variant(s) which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. One skilled in the art can use any information known about  
10 the binding or active site of the polypeptide in the selection of possible mutation sites.

One method for identifying amino acid residues or regions for mutagenesis of a protein is called "alanine scanning mutagenesis", as described by Cunningham and Wells (1989), Science, 244:1081-1085, the disclosure of which is hereby incorporated by reference. In this method, an amino acid residue or group of target residues is identified (e.g., charged residues such as Arg, Asp, His, Lys and Glu) and replaced by a neutral or negatively-charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains/residues demonstrating functional sensitivity to the substitutions are then refined by introducing additional or alternate residues at the sites of substitution. Thus, the site for introducing an amino acid sequence modification is predetermined. To optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted and the variant(s) may be screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites in which particular amino acid residues within a parent molecule are substantially different from other species or other

family members in terms of side-chain bulk, charge and/or hydrophobicity. Other sites of interest include those in which particular residues of a parent molecule are identical among other species or other family 5 members, as such positions are generally important for the biological activity of a protein.

Other sites of interest include those in which particular residues are similar or identical with proteins with similar structure or activity to the 10 parent molecule. For sTNFR-I, for example, information has been elucidated relevant to sTNFR-I-like molecules (Banner *et al.* (1993), supra, and Fu *et al.* (1995), Protein Engineering, 8(12):1233-1241). Residues Tyr<sup>9</sup>, Thr<sup>39</sup>, His<sup>55</sup> in Domain 1, residues Phe<sup>49</sup>, Ser<sup>63</sup>, Asp<sup>82</sup> in 15 Domain 2 and residues Tyr<sup>92</sup> and Ser<sup>107</sup> in Domain 3 have been identified as being potentially important for the stabilization of the structure of Domains 1, 2 and 3, respectively. Residues Pro<sup>12</sup> and His<sup>55</sup> have been identified as potentially interacting with Ser<sup>86</sup>-Tyr<sup>87</sup> 20 on subunit C of TNF- $\alpha$ . Residues Glu<sup>45</sup>-Phe<sup>49</sup> have been identified as being in a loop which potentially interacts with residues Leu<sup>29</sup>-Arg<sup>32</sup> of TNF- $\alpha$  subunit A. Residues Gly<sup>48</sup> has been identified as potentially interacting with Asn<sup>19</sup>-Pro<sup>20</sup> on subunit A of TNF- $\alpha$ . 25 Residue His<sup>58</sup>-Leu<sup>60</sup> have been identified as being in an extended strand conformation and side chain interactions with residues Arg<sup>31</sup>-Ala<sup>33</sup> on subunit A of TNF- $\alpha$  have been potentially identified with residue His<sup>58</sup> of sTNFR-I specifically interacting with residue 30 Arg<sup>31</sup>. Residues Lys<sup>64</sup>-Arg<sup>66</sup> have been identified as being in an extended strand conformation and have been identified as having side chain and main chain

interactions with residues Ala<sup>145</sup>-Glu<sup>146</sup> and residue Glu<sup>46</sup> on subunit A of TNF- $\alpha$ . Residue Met<sup>69</sup> has been identified as potentially interacting with residue Tyr<sup>115</sup> on subunit A of TNF- $\alpha$ . Residues His<sup>94</sup>-Phe<sup>101</sup> have  
5 been identified as forming a loop which interacts with residues Thr<sup>72</sup>-Leu<sup>75</sup> and Asn<sup>137</sup> of subunit C of TNF- $\alpha$ , with residue Trp<sup>96</sup> of sTNFR-I specifically interacting with residues Ser<sup>71</sup>-Thr<sup>72</sup> on subunit C of TNF- $\alpha$ , Leu<sup>100</sup> of sTNFR-I being in close proximity with residue Asn<sup>137</sup>  
10 on subunit C of TNF- $\alpha$  and residue Gln<sup>102</sup> of sTNFR-I specifically interacting with residue Pro<sup>113</sup> on subunit A of TNF- $\alpha$ .

In addition to the cysteines forming the 3 pairs of disulfide bonds within each of the four domains of  
15 the molecule, there are several other conserved residues that contribute to the stabilization of the tertiary fold of each domain.

There are two main classes into which these stabilizing residues fall. The first type contributes  
20 to the shielding of the disulfide bond sulfur atoms from solvent. An example of this residues in domain 3 is Tyr<sup>92</sup>. In domain 4 Phe<sup>133</sup> helps to shield the Cys<sup>128</sup>-Cys<sup>139</sup> disulfide bond. All four domains have either a Tyr or Phe at these same structurally conserved  
25 locations. The second class of stabilizing residues form hydrogen bonds within their respective domains. Within domain 3 Asn<sup>123</sup> and Ser<sup>107</sup> form a hydrogen bond and Ser<sup>107</sup> forms an additional hydrogen bond with Thr<sup>124</sup>. For domain 4 these residues include Asn<sup>144</sup> and  
30 Ser<sup>141</sup>.

In addition there are hydrogen bonds between domain 3 and 4 that are not seen between other domains.

These hydrogens bonds are (1) Asn<sup>105</sup> main-chain oxygen and Asn<sup>137</sup> side-chain nitrogen and (2) Ser<sup>107</sup> side-chain oxygen and Asn<sup>137</sup> main-chain nitrogen.

Another useful tool in identifying sites suitable for substitution is molecular modeling. One example of this technique is OPG. Using the homology between OPG and the extracellular ligand binding domains of TNF receptor family members, a three-dimensional model of OPG was generated based upon the known crystal structure of the extracellular domain of TNFR-I (see Example 6). This model was used to identify those residues within OPG which may be important for biological activity. Cysteine residues that are involved in maintaining the structure of the four cysteine-rich domains were identified. The following disulfide bonds were identified in the model: Domain 1: cys41 to cys54, cys44 to cys62, tyr23 and his 66 may act to stabilize the structure of this domain; Domain 2: cys65 to cys80, cys83 to cys98, cys87 to cys105; Domain 3: cys107 to cys118, cys124 to cys142; Domain 4: cys145 to cys160, cys166 to cys185. Residues were also identified which were in close proximity to TNF $\beta$  as shown in Figures 11 and 12A-12B. In this model, it is assumed that OPG binds to a corresponding ligand; TNF $\beta$  was used as a model ligand to simulate the interaction of OPG with its ligand. Based upon this modeling, the following residues in OPG may be important for ligand binding: glu34, lys43, pro66 to gln91 (in particular, pro66, his68, tyr69, tyr70, thr71, asp72, ser73, his76, ser77, asp78, glu79, leu81, tyr82, pro85, val86, lys88, glu90 and gln91), glu153 and ser155.

Alterations in these amino acid residues, either singly or in combination, may alter the biological activity of OPG. For example, changes in specific cysteine residues may alter the structure of individual

cysteine-rich domains, whereas changes in residues important for ligand binding may affect physical interactions of OPG with ligand. Structural models can aid in identifying analogs which have more desirable  
5 properties, such as enhanced biological activity, greater stability, or greater ease of formulation.

A skilled artisan will appreciate that initially sites should be modified by substitution in a relatively conservative manner. Such conservative  
10 substitutions are shown in Table 1 under the heading of "Preferred Substitutions". If such substitutions result in a change in biological activity, then more substantial changes (Exemplary Substitutions) may be introduced and/or other additions/deletions may be made  
15 and the resulting products screened.

TABLE 1: Amino Acid Substitutions

<u>Original Residue</u>	<u>Preferred Substitutions</u>	<u>Exemplary Substitutions</u>
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; norleucine

In making such changes, the hydropathic index of amino acids may be considered. The importance of the 5 hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle (1982), J.

Mol. Biol., 157:105-131, the disclosure of which is incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and 5 still retain a similar biological activity.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the functionally equivalent protein 10 or peptide thereby created is intended for use in immunological embodiments, as in the present case. U.S. Patent 4,554,101, the disclosure of which is incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as 15 governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with a biological property of the protein.

U.S. Patent 4,554,101 also teaches the 20 identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in U.S. Patent 4,554,101 a skilled artisan would be able to identify epitopes, for example, within the amino acid sequence of an 25 STNFR. These regions are also referred to as "epitopic core regions". Numerous scientific publications have been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou and Fasman (1974), 30 Biochemistry, 13(2):222-245; Chou and Fasman (1974), Biochemistry, 13(2):211-222; Chou and Fasman (1978), Adv. Enzymol. Relat. Areas Mol. Biol., 47:45-148; Chou and Fasman (1978), Ann. Rev. Biochem., 47:251-276 and Chou and Fasman (1979), Biophys. J., 26:367-384, the 35 disclosures of which are incorporated herein by reference). Moreover, computer programs are currently

available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson and Wolf (1988), Comput. Appl. Biosci., 5 4(1):181-186 and Wolf et al. (1988), Comput. Appl. Biosci., 4(1):187-191, the disclosures of which are incorporated herein by reference); the program PepPlot® (Brutlag et al. (1990), CABS, 6:237-245 and Weinberger et al. (1985), Science, 228:740-742, the disclosures of 10 which are incorporated herein by reference); and other programs for protein tertiary structure prediction (Fetrow and Bryant (1993), BIOTECHNOLOGY, 11:479-483, the disclosure of which is incorporated herein by reference).

15 In contrast, substantial modifications in the functional and/or chemical characteristics of a parent molecule may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide 20 backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the relative charge or hydrophobicity of the protein at the target site or (c) the bulk of the side chain. Naturally-occurring residues are divided into groups based on 25 common side chain properties:

- 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr;
- 3) acidic: Asp, Glu;
- 4) basic: Asn, Gln, His, Lys, Arg;
- 5) aromatic: Trp, Tyr, Phe; and
- 6) residues that influence chain orientation: Gly, Pro.

30 Non-conservative substitutions may involve the exchange of a member of one of these groups for 35

another. For example, substituted residues may be introduced into regions of OPG or the sTNFRs that are homologous with other NGF/TNF receptor family members or into non-homologous regions of the protein.

5 A variety of amino acid substitutions or deletions may be made to modify or add N-linked or O-linked glycosylation sites, resulting in a protein with altered glycosylation. The sequence may be modified to add glycosylation sites to or to delete N-linked or O-linked glycosylation sites from the parent molecule. An 10 asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either 15 Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. In the 30 kDa TNF inhibitor, for example, proven or predicted asparagine residues exist at positions 14, 105 and 111.

Specific mutations of the sequences of the parent 20 molecules may involve substitution of a non-native amino acid at the amino-terminus, carboxy-terminus or at any site of the protein that is modified by the addition of an N-linked or O-linked carbohydrate. Such modifications may be of particular utility in the 25 addition of an amino acid (e.g., cysteine), which is advantageous for the linking of a water-soluble polymer to form a derivative. For example, WO 92/16221 describes the preparation of sTNFR-I muteins, e.g., wherein an asparagine residue at position 105 of the 30 native human protein is changed to cysteine (c105 sTNFR-I).

In a specific embodiment, a variant polypeptide will preferably be substantially homologous to the amino acid of the parent molecule from which it is 35 derived. The term "substantially homologous" as used herein means a degree of homology that is in excess of

80%, preferably in excess of 90%, more preferably in excess of 95% or most preferably even 99%. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment, as set forth by Dayhoff (1972), Atlas of Protein Sequence and Structure, 5:124, National Biochemical Research Foundation, Washington, D.C., the disclosure of which is hereby incorporated by reference. Also included within the term "substantially homologous" are variant(s) of parent molecules that may be isolated by cross-reactivity with antibodies to the parent molecule amino acid sequences or whose genes may be isolated through hybridization with the DNA of parent molecules or segments thereof.

#### Polypeptide Derivatives

This invention also comprises chemically modified derivatives of the parent molecule(s) in which the protein is linked to a nonproteinaceous moiety (e.g., a polymer) in order to modify properties. These chemically modified parent molecules are referred to herein as "derivatives". Such derivatives may be prepared by one skilled in the art given the disclosures herein. Conjugates may be prepared using glycosylated, non-glycosylated or de-glycosylated parent molecule(s) and suitable chemical moieties. Typically non-glycosylated parent molecules and water-soluble polymers will be used. Other derivatives encompassed by the invention include post-translational modifications (e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, and chemical modifications of N-linked or O-

linked carbohydrate chains. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

5        Water-soluble polymers are desirable because the protein to which each is attached will not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a  
10      therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically and, if so, the therapeutic profile of the protein (e.g., duration of  
15      sustained release; resistance to proteolysis; effects, if any, on dosage; biological activity; ease of handling; degree or lack of antigenicity and other known effects of a water-soluble polymer on a therapeutic proteins).

20       Suitable, clinically acceptable, water-soluble polymers include but are not limited to polyethylene glycol (PEG), polyethylene glycol propionaldehyde, copolymers of ethylene glycol/propylene glycol, monomethoxy-polyethylene glycol,  
25      carboxymethylcellulose, dextran, polyvinyl alcohol (PVA), polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, poly ( $\beta$ -amino acids) (either homopolymers or random copolymers), poly(n-vinyl  
30      pyrrolidone)polyethylene glycol, polypropylene glycol homopolymers (PPG) and other polyalkylene oxides, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols (POG) (e.g., glycerol) and other polyoxyethylated polyols, polyoxyethylated sorbitol, or polyoxyethylated glucose, colonic acids or  
35

other carbohydrate polymers, Ficoll or dextran and mixtures thereof. As used herein, polyethylene glycol is meant to encompass any of the forms that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

5 Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The water-soluble polymers each may be of any molecular weight and may be branched or unbranched.

10 Generally, the higher the molecular weight or the more branches, the higher the polymer:protein ratio. The water-soluble polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa (the term "about" indicating that in preparations

15 of a water-soluble polymer, some molecules will weigh more, some less, than the stated molecular weight). The average molecular weight of each water-soluble polymer preferably is between about 5 kDa and about 40 kDa, more preferably between about 10kDa and about 35 kDa and most preferably between about 15kDa and about 30 kDa.

There are a number of attachment methods available to those skilled in the art, including acylation reactions or alkylation reactions (preferably to generate an amino-terminal chemically modified protein) with a reactive water-soluble molecule. See, for example, EP 0 401 384; Malik *et al.* (1992), Exp. Hematol., 20:1028-1035; Francis (1992), Focus on Growth Factors, 3(2):4-10, published by Mediscript, Mountain Court, Friern Barnet Lane, London N20 0LD, UK; EP 0 154 316; EP 0 401 384; WO 92/16221; WO 95/34326; WO 95/13312; WO 96/11953; WO 96/19459 and WO 96/19459 and the other publications cited herein that relate to pegylation, the disclosures of which are hereby incorporated by reference.

Pegylation also may be specifically carried out using water-soluble polymers having at least one reactive hydroxy group (e.g. polyethylene glycol). The water-soluble polymer can be reacted with an activating group, thereby forming an "activated linker" useful in modifying various proteins. The activated linkers can be monofunctional, bifunctional, or multifunctional.

Activating groups which can be used to link the water-soluble polymer to two or more proteins include the following: sulfone, maleimide, sulfhydryl, thiol, triflate, tresylate, azidirine, oxirane and 5-pyridyl. Useful reagents having a reactive sulfone group that can be used in the methods include, without limitation, chlorosulfone, vinylsulfone and divinylsulfone. These PEG derivatives are stable against hydrolysis for extended periods in aqueous environments at pHs of about 11 or less, and can form linkages with molecules to form conjugates which are also hydrolytically stable. Useful homobifunctional derivatives are PEG-bis-chlorosulfone and PEG-bis-vinylsulfone (see WO 95/13312).

WO 97/04003, the disclosure of which is hereby incorporated by reference, teaches methods of making sulfone-activated linkers by obtaining a compound having a reactive hydroxyl group and converting the hydroxyl group to a reactive Michael acceptor to form an activated linker, with tetrahydrofuran as the solvent for the conversion. The application also teaches a process for purifying the activated linkers which utilizes hydrophobic interaction chromatography to separate the linkers based on size and end-group functionality.

As an example, chemically modified derivatives of OPG may provide such advantages as increased stability, increased time in circulation, or decreased immunogenicity (see U.S. Patent No. 4,179,337). The

chemical moieties for derivitization may be selected from water-soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemically modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

#### Polyvalent Forms

Polyvalent forms, i.e., molecules comprising more than one active moiety, may be constructed. In one embodiment, an sTNFR variant may possess multiple tumor necrosis factor binding sites for the TNF ligand.

Additionally, the molecule may possess at least one tumor necrosis factor binding site and, depending upon the desired characteristic of polyvalent form, at least one site of another molecule (e.g., a TNF- $\alpha$  inhibitor(s), and an OPG).

Active moieties may be linked using conventional coupling techniques (see WO 92/16221, WO 95/13312 and WO 95/34326, the disclosures of which are hereby incorporated by reference). For example, WO 92/16221 and WO 95/34326 describe the preparation of various dimerized sTNFR-I molecules, e.g., dimerized c105 sTNFR-I. Techniques for formation of polyvalent forms include photochemical crosslinking (e.g., exposure to ultraviolet light), chemical crosslinking (e.g., with bifunctional linker molecules such as polyethylene glycol), and mutagenesis (e.g., introduction of additional cysteine residues).

Polyvalent forms may be constructed by chemically coupling at least one parent molecule and another moiety with any clinically accepted linker (e.g., a water-soluble polymer). In principle, the linker must not impart new immunogenicity. The linker also must not, by virtue of the new amino acid residues, alter the hydrophobicity and charge balance of the structure, which affects its biodistribution and clearance. A variety of chemical crosslinkers may be used depending upon which properties of the protein dimer are desired. For example, crosslinkers may be short and relatively rigid or longer and more flexible, may be biologically reversible, and may provide reduced immunogenicity or longer pharmacokinetic half-life.

In one example, OPG molecules are linked through the amino terminus by a two step synthesis (see Example 12). In the first step, OPG is chemically modified at the amino terminus to introduce a protected thiol,

which after purification is deprotected and used as a point of attachment for site-specific conjugation through a variety of crosslinkers with a second OPG molecule. Amino-terminal crosslinks include, but are  
5 not limited to, a disulfide bond, thioether linkages using short-chain, bis-functional aliphatic crosslinkers, and thioether linkages to variable length, bifunctional polyethylene glycol crosslinkers (PEG "dumbbells"). Also encompassed by PEG dumbbell  
10 synthesis of OPG dimers is a byproduct of such synthesis, termed a "monobell". An OPG monobell consists of a monomer coupled to a linear bifunctional PEG with a free polymer terminus. Alternatively, OPG may be crosslinked directly through a variety of amine  
15 specific homobifunctional crosslinking techniques which include reagents such as: diethylenetriaminepentaacetic dianhydride (DTPA), p-benzoquinone (pBQ) or bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>) as well as others known in the art. It is also possible to thiolate OPG  
20 directly with reagents such as iminothiolane in the presence of a variety of bifunctional, thiol specific crosslinkers, such as PEG bismaleimide, and achieve dimerization and/or dumbbells in a one step process.

The water-soluble polymers for this polyvalent form can be, based on the monomers listed herein, homopolymers, random or block copolymers, terpolymers straight chain or branched, substituted or unsubstituted. The polymer can be of any length or molecular weight, but these characteristics can affect  
25 the biological properties. Polymer average molecular weights particularly useful for decreasing clearance rates in pharmaceutical applications are in the range of 2,000 to 35,000 daltons. In addition, the length of the polymer can be varied to optimize or confer the  
30 desired biological activity.  
35

Alternatively, a bivalent molecule may consist of two tandem repeats of parent molecules separated by a polypeptide linker region. The design of the polypeptide linkers is similar in design to the 5 insertion of short loop sequences between domains in the de novo design of proteins (Mutter (1988), TIBS, 13:260-265 and Regan and DeGrado (1988), Science, 241:976-978, the disclosures of which are hereby incorporated by reference). Several different linker 10 constructs have been assembled and shown to be useful for forming single chain antibodies; the most functional linkers vary in size from 12 to 25 amino acids (amino acids having unreactive side groups, e.g., alanine, serine and glycine) which together constitute 15 a hydrophilic sequence, have a few oppositely charged residues to enhance solubility and are flexible (Whitlow and Filpula (1991), Methods: A Companion to Methods in Enzymology, 2:97-105; and Brigido et al. (1993), J. Immunol., 150:469-479, the disclosures of 20 which are hereby incorporated by reference). It has been shown that a linker suitable for single chain antibodies is effective to produce a dimeric form of the human sTNFR-II (Neve et al. (1996), Cytokine, 8(5):365-370, the disclosure of which is hereby 25 incorporated by reference).

Self-associating variants are another example of polyvalent forms. Such self-associating variants may be bound covalently (typically by disulfide bonds) or noncovalently. Analysis of carboxy-terminal deletions 30 of OPG, for example, suggest that at least a portion of the region 186-401 is involved in association of OPG polypeptides. Substitution of part or all of the region of OPG amino acids 186-401 with an amino acid sequence capable of self-association is also encompassed by the 35 invention.

Polyvalent forms may also be formed using substitution variants. Parent molecules may be modified to form dimers or multimers by site-directed mutagenesis to create unpaired cysteine residues for 5 interchain disulfide bond formation.

Additionally, a parent molecule may be chemically coupled to biotin, and the resulting conjugate may then be allowed to bind to avidin, resulting in tetravalent avidin/biotin/parent molecules. A parent molecule may 10 also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugates precipitated with anti-DNP or anti-TNP-IgM to form decameric conjugates.

In yet another embodiment, recombinant fusion 15 proteins may also be produced wherein each recombinant chimeric molecule has a parent molecule(s) sequence amino-terminally or carboxy-terminally fused to all or part of the constant domains, but at least one constant domain, of the heavy or light chain of human 20 immunoglobulin. For example, a chimeric TNF- $\alpha$  inhibitor(s)/IgG1 (or IgG1/TNF- $\alpha$  inhibitor(s)) fusion protein may be produced from a light chain-containing chimeric gene: a TNF- $\alpha$  inhibitor(s)/human kappa light chain chimera (TNF- $\alpha$  inhibitor(s)/Ck) or a human kappa 25 light chain/TNF- $\alpha$  inhibitor(s) chimera (Ck/TNF- $\alpha$  inhibitor(s)); or a heavy chain-containing chimeric gene: a TNF- $\alpha$  inhibitor(s)/human gamma-1 heavy chain chimera (TNF- $\alpha$  inhibitor(s)/Cg-1) or a human gamma-1 30 heavy chain/TNF- $\alpha$  inhibitor(s) chimera (Cg-1/TNF- $\alpha$  inhibitor(s)). Alternatively, an OPG-Fc chimera may be formed as described in WO 97/23614, which is hereby incorporated by reference. Following transcription and translation of a heavy-chain chimeric gene, or of a light chain-containing gene and a heavy-chain chimeric

gene, the gene products may be assembled into a single chimeric molecule having a parent molecule(s) displayed bivalently. Additional details relating to the construction of such chimeric molecules are disclosed  
5 in United States Patent 5,116,964, WO 89/09622, WO 91/16437, WO 97/23614 and EP 315062, the disclosures of which are hereby incorporated by reference.

In yet a further embodiment, recombinant fusion proteins may also be produced wherein each recombinant  
10 chimeric molecule has at least one TNF- $\alpha$  inhibitor(s), as described herein, and at least a portion of the region 186-401 of osteoprotogerin or a variant thereof, as described in European Patent Application No. 96309363.8, the disclosures of which are hereby  
15 incorporated by reference. Either the TNF- $\alpha$  inhibitor(s) or the portion of osteoprotogerin may be at the amino-terminus or the carboxy-terminus of the chimeric molecule.

#### Nucleic Acids

20 The invention provides for an isolated nucleic acid encoding a polypeptide having at least one of the biological activities of OPG. As described herein, the biological activities of OPG include, but are not limited to, any activity involving bone metabolism and  
25 in particular, include increasing bone density. The nucleic acids of the invention are selected from the following:

- a) the nucleic acid sequences as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) or complementary strands thereof;
- 30 b) the nucleic acids which hybridize under stringent conditions with the polypeptide-encoding region in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124); and

c) nucleic acids which hybridize under stringent conditions with nucleotides 148 through 337 inclusive as shown in Figure 1A.

5 d) the nucleic acid sequences which are degenerate to the sequences in (a) and (b).

The invention provides for nucleic acids which encode rat, mouse and human OPG as well as nucleic acid sequences hybridizing thereto which encode a polypeptide having at least one of the biological 10 activities of OPG. Also provided for are nucleic acids which hybridize to a rat OPG EST encompassing nucleotides 148-337 as shown in Figure 1A. The conditions for hybridization are generally of high stringency such as 5xSSC, 50% formamide and 42°C 15 described in Example 1 of the specification. Equivalent stringency to these conditions may be readily obtained by adjusting salt and organic solvent concentrations and temperature. The nucleic acids in (b) encompass sequences encoding OPG-related polypeptides which do 20 not undergo detectable hybridization with other known members of the TNF receptor superfamily. In a preferred embodiment, the nucleic acids are as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124).

25 The length of hybridizing nucleic acids of the invention may be variable since hybridization may occur in part or all of the polypeptide-encoding regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), and may also occur 30 in adjacent noncoding regions. Therefore, hybridizing nucleic acids may be truncations or extensions of the sequences shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124). Truncated or extended nucleic acids are encompassed by the 35 invention provided they retain one or more of the biological properties of OPG. The hybridizing nucleic

acids may also include adjacent noncoding regions which are 5' and/or 3' to the OPG coding region. The noncoding regions include regulatory regions involved in OPG expression, such as promoters, enhance,  
5 translational initiation sites, transcription termination sites and the like.

Hybridization conditions for nucleic acids are described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed. Cold Spring Harbor Laboratory  
10 Press, Cold Spring Harbor, New York (1989)

DNA encoding rat OPG was provided in plasmid pMO-B1.1 deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under ATCC accession no. 69970. DNA encoding mouse OPG was  
15 provided in plasmid pRcCMV-murine OPG deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under accession no. 69971. DNA encoding human OPG was provided in plasmid pRcCMV - human OPG deposited with the American Type Culture  
20 Collection, Rockville, MD on December 27, 1995 under accession no. 69969. The nucleic acids of the invention will hybridize under stringent conditions to the DNA inserts of ATCC accession nos. 69969, 69970, and 69971 and have at least one of the biological activities of  
25 OPG.

Also provided by the invention are derivatives of the nucleic acid sequences as shown in Figures 2B, 9A and 9B. As used herein, derivatives include nucleic acid sequences having addition, substitution, insertion  
30 or deletion of one or more residues such that the resulting sequences encode polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting polypeptide has the activity of OPG. The nucleic acid  
35 derivatives may be naturally occurring, such as by splice variation or polymorphism, or may be constructed

using site-directed mutagenesis techniques available to the skilled worker. One example of a naturally occurring variant of OPG is a nucleic acid encoding a lys to asn change at residue 3 within the leader sequence (see Example 5). It is anticipated that nucleic acid derivatives will encode amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other derivatives include a nucleic acid encoding a membrane-bound form of OPG having an extracellular domain as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) along with transmembrane and cytoplasmic domains.

In one embodiment, derivatives of OPG include nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the carboxy terminus. Nucleic acids encoding OPG may have from 1 to 216 amino acids deleted from the carboxy terminus. Optionally, an antibody Fc region may extend from the new carboxy terminus to yield a biologically active OPG-Fc fusion polypeptide. (see Example 11). In preferred embodiments, nucleic acids encode OPG having the amino acid sequence from residues 22-185, 22-189, 22-194 or 22-201 (using numbering in Figure 9E-F) and optionally, encoding an Fc region of human IgG.

Also included are nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the amino terminus. Truncated forms include those lacking part or all the 21 amino acids comprising the leader sequence. Additionally, the invention provides for nucleic acids encoding OPG having from 1 to 10 amino acids deleted from the mature amino terminus (at residue 22) and ,optionally, having from 1 to 216 amino acids deleted from the carboxy terminus (at residue 401). Optionally, the nucleic acids may encode a methionine residue at the amino terminus. Examples of

such OPG truncated polypeptides are described in Example 8.

Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing OPG. In humans, tissue sources for OPG include kidney, liver, placenta and heart. Genomic DNA encoding OPG is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to reconstitute part or all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of interferon genes). RNA is obtained most easily by prokaryotic expression vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Nucleic acid sequences of the invention are used for the detection of OPG sequences in biological samples in order to determine which cells and tissues are expressing OPG mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to OPG. Such screening is well within the capabilities of one skilled in the art using appropriate hybridization conditions to detect homologous sequences. The nucleic acids are also useful for modulating the expression of OPG levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity (see Example 3).

#### Vectors and Host Cells

Expression vectors containing nucleic acid sequences encoding OPG, host cells transformed with

said vectors and methods for the production of OPG are also provided by the invention. An overview of expression of recombinant proteins is found in Methods of Enzymology v. 185, Goeddel, D.V. ed. Academic Press 5 (1990).

Host cells for the production of OPG include prokaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression. 10 Preferred mammalian host cells include COS, CHOD-, 293, CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for OPG activity. 15 Mammalian expression allows for the production of secreted polypeptides which may be recovered from the growth medium.

Vectors for the expression of OPG contain at a minimum sequences required for vector propagation and 20 for expression of the cloned insert. These sequences include a replication origin, selection marker, promoter, ribosome binding site, enhancer sequences, RNA splice sites and transcription termination site. Vectors suitable for expression in the aforementioned 25 host cells are readily available and the nucleic acids of the invention are inserted into the vectors using standard recombinant DNA techniques. Vectors for tissue-specific expression of OPG are also included. Such vectors include promoters which function 30 specifically in liver, kidney or other organs for production in mice, and viral vectors for the expression of OPG in targeted human cells.

Using an appropriate host-vector system, OPG is produced recombinantly by culturing a host cell 35 transformed with an expression vector containing nucleic acid sequences encoding OPG under conditions

such that OPG is produced, and isolating the product of expression. OPG is produced in the supernatant of transfected mammalian cells or in inclusion bodies of transformed bacterial host cells. OPG so produced may 5 be purified by procedures known to one skilled in the art as described below. The expression of OPG in mammalian and bacterial host systems is described in Examples 7 and 8. Expression vectors for mammalian hosts are exemplified by plasmids such as pDSR $\alpha$  10 described in PCT Application No. 90/14363. Expression vectors for bacterial host cells are exemplified by plasmids pAMG21 and pAMG22-His described in Example 8. Plasmid pAMG21 was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 15 under accession no. 98113. Plasmid pAMG22-His was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98112. It is anticipated that the specific plasmids and host cells described are for illustrative purposes and 20 that other available plasmids and host cells could also be used to express the polypeptides.

The invention also provides for expression of OPG from endogenous nucleic acids by in vivo or ex vivo recombination events to allow modulation of OPG from 25 the host chromosome. Expression of OPG by the introduction of exogenous regulatory sequences (e.g. promoters or enhancers) capable of directing the production of OPG from endogenous OPG coding regions is also encompassed. Stimulation of endogenous regulatory 30 sequences capable of directing OPG production (e.g. by exposure to transcriptional enhancing factors) is also provided by the invention.

#### Antibodies

Also encompassed by the invention are antibodies 35 specifically binding to OPG. Antigens for the generation of antibodies may be full-length

polypeptides or peptides spanning a portion of the OPG sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with OPG are known to one skilled in the art (see, for example, Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the art. Also encompassed are human antibodies made in mice.

Anti-OPG antibodies of the invention may be used as an affinity reagent to purify OPG from biological samples (see Example 10). In one method, the antibody is immobilized on CNBr-activated Sepharose and a column of antibody-Sepharose conjugate is used to remove OPG from liquid samples. Antibodies are also used as diagnostic reagents to detect and quantitate OPG in biological samples by methods described below.

Pharmaceutical compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of a polypeptide comprising OPG or the other therapeutic molecules used (e.g., IL-1ra, sTNF-RI, or SLPI) together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. Two or more of the therapeutic

molecules (e.g., OPG, IL-1ra, sTNF-RI, or SLPI) can be formulated together or packaged together in a kit. The term "therapeutically effective amount" means an amount which provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal or benzyl alcohol, and antioxidants such as ascrobic acid or sodium metabisulfite. Also encompassed are compositions comprising any of the therapeutic molecules modified with water-soluble polymers to increase solubility or stability. Compositions may also comprise incorporation of any of the therapeutic molecules into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time.

Specifically, compositions herein may comprise incorporation into polymer matrices such as hydrogels, silicones, polyethylenes, ethylene-vinyl acetate copolymers, or biodegradable polymers. Examples of hydrogels include polyhydroxyalkylmethacrylates (p-HEMA), polyacrylamide, polymethacrylamide, polyvinylpyrrolidone, polyvinyl alcohol and various polyelectrolyte complexes. Examples of biodegradable polymers include polylactic acid (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA, polyamides and copolymers of polyamides and polyesters. Other controlled release formulations include microcapsules, microspheres, macromolecular complexes and polymeric beads which may be administered by injection.

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the

pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18<sup>th</sup> ed. A.R. Gennaro, ed. Mack, Easton, PA 5 (1980).

Compositions of the invention may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled in the art. 10

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together 15 with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the therapeutic molecule coding region to cells and tissues as part of an anti-sense or gene therapy regimen.

20 Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, 25 osteocytes, osteoblasts and osteoclasts, are involved in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of bone tissue whereas osteoclasts are associated with resorption. Resorption, or the dissolution of bone 30 matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal tissue and in resorption induced by hormones. For 35 instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing

concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to 5 parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone formation and bone resorption. Peak bone mass occurs after skeletal maturity prior to the fourth decade. 10 Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts earlier in females than males and is distinctly accelerated after menopause in some females 15 (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels. Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone 20 destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in 25 bone resorption with a normal rate of bone formation. About 25 to 30 percent of all white females in the United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric 30 fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been 35 identified which may contribute to the condition. They include alteration in hormone levels accompanying aging

and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard 5 the process. To date, however, an effective treatment for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of OPG. The bone disorder may be any disorder 10 characterized by a net bone loss (osteopenia or osteolysis). In general, treatment with OPG is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption 15 rate is above normal or to reduce bone resorption to below normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with OPG include the following:

- 20 • Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.
- 25 • Paget's disease of bone (osteitis deformans) in adults and juveniles
- 30 • Osteomyelitis, or an infectious lesion in bone, leading to bone loss.
- Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignancies (multiple myeloma, lymphoma and leukemia),
- 35 • idiopathic hypercalcemia, and hypercalcemia

associated with hyperthyroidism and renal function disorders.

- Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic and renal diseases.
- Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus, rheumatoid arthritis, periodontal disease, osteolytic metastasis, and other conditions

It is understood that OPG may be used alone or in conjunction with other factors for the treatment of bone disorders. In one embodiment, osteoprotegerin is used in conjunction with a therapeutically effective amount of a factor which stimulates bone formation. Such factors include but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12; transforming growth factor- $\beta$  (TGF- $\beta$ ) and TGF- $\beta$  family members; interleukin-1 (IL-1) inhibitors; TNF $\alpha$  inhibitors; parathyroid hormone and analogs thereof, parathyroid related protein and analogs thereof; E series prostaglandins; bisphosphonates (such as alendronate and others); bone-enhancing minerals such as fluoride and calcium; non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors, such as Celebrex™ and Vioxx™; immunosuppressants, such as methotrexate or leflunomide; serine protease inhibitors such as secretory leukocyte protease inhibitor (SLPI); IL-6 inhibitors (e.g., antibodies to IL-6), IL-8 inhibitors (e.g., antibodies to IL-8); IL-18 inhibitors (e.g., IL-18 binding protein or IL-18 antibodies); Interleukin-1 converting enzyme (ICE) modulators; fibroblast growth

factors FGF-1 to FGF-10 and FGF modulators; PAF antagonists; keratinocyte growth factor (KGF), KGF-related molecules, or KGF modulators; matrix metalloproteinase (MMP) modulators; Nitric oxide synthase (NOS) modulators, including modulators of inducible NOS; modulators of glucocorticoid receptor; modulators of glutamate receptor; modulators of lipopolysaccharide (LPS) levels; and noradrenaline and modulators and mimetics thereof.

10       The invention also relates to treatment of IL-1 mediated disease by treatment with an IL-1 inhibitor in conjunction with a serine protease inhibitor. In particular, this method is useful for treatment of asthma and rheumatoid arthritis.

15       The invention relates further to treatment of TNF-mediated disease by treatment with a TNF inhibitor in conjunction with a serine protease inhibitor. In particular, this method is useful for treatment of rheumatoid arthritis.

20       In preferred embodiments, a polypeptide comprising OPG is used in conjunction with particular therapeutic molecules to treat various inflammatory conditions, autoimmune conditions, and other conditions leading to bone loss. Depending on the condition and the desired level of treatment, two, three, or more agents may be administered. These agents may be provided together by inclusion in the same formulation or inclusion in a treatment kit, or they may be provided separately. When administered by gene therapy, the genes encoding the protein agents may be included in the same vector, optionally under the control of the same promoter region, or in separate vectors. Particularly preferred molecules in the aforementioned classes are as follows.

- IL-1 inhibitors: IL-1ra proteins and soluble IL-1 receptors. The most preferred IL-1 inhibitor is anakinra.
- TNF- $\alpha$  inhibitors: soluble tumor necrosis factor receptor type I (sTNF-RI; -RI is also called the p55 receptor); soluble tumor necrosis factor receptor type II (also called the p75 receptor); and monoclonal antibodies that bind the TNF receptor. Most preferred is sTNF-RI as described in WO 98/24463, etanercept (Enbrel $^{\circ}$ ), and Avakine $^{\circ}$ . Exemplary TNF- $\alpha$  inhibitors are described in EP 422 339, EP 308 378, EP 393 438, EP 398 327, and EP 418 014.
- serine protease inhibitors: SLPI, ALP, MPI, HUSI-I, BMI, and CUSI. These inhibitors also may be viewed as exemplary LPS modulators, as SLPI has been shown to inhibit LPS responses. Jin *et al.* (1997), *Cell* 88(3): 417-26 (incorporated by reference).

Particularly preferred methods of treatment concern use of TNF- $\alpha$  inhibitors and IL-1 inhibitors in conjunction with polypeptides comprising OPG. Such polypeptides may be used with either or both TNF- $\alpha$  inhibitors and IL-1 inhibitors for treatment of conditions such as rheumatoid arthritis and multiple sclerosis.

The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

**EXAMPLE 1**  
Identification and isolation of the rat OPG cDNA  
Materials and methods for cDNA cloning and analysis are described in Maniatis *et al*, *ibid*. Polymerase chain reactions (PCR) were performed using a Perkin-Elmer 9600 thermocycler using PCR reaction mixture (Boehringer-Mannheim) and primer concentrations

specified by the manufacturer. In general, 25-50 µl reactions were denatured at 94°C, followed by 20-40 cycles of 94°C for 5 seconds, 50-60°C for 5 seconds, and 72°C for 3-5 minutes. Reactions were then treated 5 for 72 °C for 3-5 minutes. Reactions were then analyzed by gel electrophoresis as described in Maniatis *et al.*, *ibid.*

A cDNA library was constructed using mRNA isolated from embryonic d20 intestine for EST analysis (Adams *et* 10 *al.* *Science* 252, 1651-1656 (1991)). Rat embryos were dissected, and the entire developing small and large intestine removed and washed in PBS. Total cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi *Anal.* 15 *Biochem.* 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the manufacturer's recommended procedures. A random primed cDNA library was prepared 20 using the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md). The random cDNA primer containing an internal Not I restriction site was used to initiate first strand synthesis and had the following sequence:

5'-AAAGGAAGGAAAAAGCGGCCGCTACANNNNNNNT-3'

25 (SEQ ID NO:1)

Not I

For the first strand synthesis three separate reactions were assembled that contained 2.5 µg of poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random 30 primer. After second strand synthesis, the reaction products were separately extracted with a mixture of phenol:choroform:isoamyl alcohol (25:24:1 ratio), and then ethanol precipitated. The double strand (ds) cDNA products of the three reactions were combined and 35 ligated to the following ds oligonucleotide adapter:

5'-TCGACCCACGGTCCG-3' (SEQ ID NO:2)

3'-GGGTGCAGGCP-5' (SEQ ID NO:3)

After ligation the cDNA was digested to completion with Not I, extracted with phenol:chloroform:isoamyl  
5 (25:24:1) alcohol and ethanol precipitated. The resuspended cDNA was then size fractionated by gel filtration using premade columns provided with the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) as recommended by the manufacturer. The two  
10 fractions containing the largest cDNA products were pooled, ethanol precipitated and then directionally ligated into Not I and Sal I digested pMOB vector DNA (Strathmann *et al*, 1991). The ligated cDNA was introduced into competent ElectroMAX DH10B E. coli  
15 (Gibco BRL, Gaithersburg, MD) by electroporation. For automated sequence analysis approximately 10,000 transformants were plated on 20cm x 20cm agar plates containing ampicillin supplemented LB nutrient media. The colonies that arose were picked and arrayed onto 96  
20 well microtiter plates containing 200 ml of L-broth, 7.5% glycerol, and 50 µg/ml ampicillin. The cultures were grown overnight at 37°C, a duplicate set of microtiter plates were made using a sterile 96 pin replicating tool, then both sets were stored at -80°C  
25 for further analysis. For full-length cDNA cloning approximately one million transformants were plated on 96 bacterial ampicillin plates containing about 10,000 clones each. The plasmid DNA from each pool was separately isolated using the Qiagen Plasmid Maxi Kit  
30 (Qiagen Corp., Germany) and arrayed into 96 microtiter plates for PCR analyses.

To sequence random fetal rat intestine cDNA clones, glycerol stocks were thawed, and small aliquots diluted 1:25 in distilled. Approximately 3.0 ul of  
35 diluted bacterial cultures were added to PCR reaction

mixture (Boehringer-Mannheim) containing the following oligonucleotides:

5'-TGTAAAACGACGGCCAGT-3' (SEQ ID NO:4)

5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO:5)

5       The reactions were incubated in a thermocycler (Perkin-Elmer 9600) with the following cycle conditions: 94°C for 2 minutes; 30 cycles of 94°C for 5 seconds, 50°C for 5 seconds, and 72°C for 3 minutes.; 72°C for 4 minutes. After incubation in the 10 thermocycler, the reactions were diluted with 2.0 mL of water. The amplified DNA fragments were further purified using Centricon columns (Princeton Separations) using the manufacturer's recommended procedures. The PCR reaction products were sequenced on 15 an Applied Biosystems 373A automated DNA sequencer using T3 primer (oligonucleotide 353-23; 5'- CAATTAACCCTCACTAAAGG-3') (SEQ ID NO:6) Taq dye-terminator reactions (Applied Biosystems) following the manufacturer's recommended procedures.

20       The resulting 5' nucleotide sequence obtained from randomly picked cDNA clones translated and then compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson *et al.* *Meth. Enzymol.* 183, (1990)).

25       Translated sequences were also analysed for the presence of a specific cysteine-rich protein motif found in all known members of the tumor necrosis factor receptor (TNFR) superfamily (Smith *et al.* (1994) *Cell* 76: 959-62), using the sequence profile 30 method of Gribskov *et al.* (1987), *Proc. Natl. Acad. Sci. USA* 83: 4355-9), as modified by Luethy *et al.* (1994), *Protein Science* 3: 139-46.

Using the FASTA and Profile search data, an EST, FRI-1 (Fetal Rat Intestine-1), was identified as a 35 possible new member of the TNFR superfamily. FRI-1

contained an approximately 600 bp insert with a LORF of about 150 amino acids. The closest match in the database was the human type II TNFR (TNFR-II). The region compared showed an about 43% homology between 5 TNFR-II and FRI-1 over this 150 aa LORF. Profile analysis using the first and second cysteine-rich repeats of the TNFR superfamily yielded a Z score of about 8, indicating that the FRI-1 gene possibly encodes a new family member.

10 To deduce the structure of the FRI-1 product, the fetal rat intestine cDNA library was screened for full length clones. The following oligonucleotides were derived from the original FRI-1 sequence:

15 5'-GCATTATGACCCAGAAACCGGAC-3' (SEQ ID NO:7)  
5'-AGGTAGCGCCCTTCCTCACATTC-3' (SEQ ID NO:8)

These primers were used in PCR reactions to screen 96 pools of plasmid DNA, each pool containing plasmid DNA from 10,000 independent cDNA clones. Approximately 1 ug of plasmid pool DNA was amplified in a PCR 20 reaction mixture (Boehringer-Mannheim) using a Perkin-Elmer 96 well thermal cycler with the following cycle conditions: 2 min at 94°C, 1 cycle; 15 sec at 94°C, then 45 sec at 65°C, 30 cycles; 7 min at 65°C, 1 cycle. PCR reaction products were analysed by gel electrophoresis. 25 13 out of 96 plasmid DNA pools gave rise to amplified DNA products with the expected relative molecular mass.

DNA from one positive pool was used to transform competent ElectroMAX DH10B *E. coli* (Gibco BRL, Gaithersburg, MD) as described above. Approximately 30 40,000 transformants were plated onto sterile nitrocellulose filters (BA-85, Schleicher and Schuell), and then screened by colony hybridization using a <sup>32</sup>P-dCTP labeled version of the PCR product obtained above. Filters were prehybridized in 5X SSC, 50% deionized 35 formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C.

Filters were then hybridized in 5X SSC, 50% deionized formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and about 5 ng/ml of labelled probe for about 18 hours at 42°C. The filters  
5 were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 minutes at 55°C, and finally in 0.5X SSC for 10-15 min at 55°C. Hybridizing clones were detected following autoradiography, and then replated onto nitrocellulose filters for secondary screening. Upon secondary  
10 screening, a plasmid clone (pB1.1) was isolated, then amplified in L-broth media containing 100 ug/ml ampicillin and the plasmid DNA obtained. Both strands of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA  
15 search of the public database to detect any existing sequence matches and/or similarities. No matches to any known genes or EST's were found, although there was an approximate 45% similarity to the human and mouse TNFR-II genes. A methionine start codon is found at bp 124  
20 of the nucleotide sequence, followed by a LORF encoding 401 aa residues that terminates at bp 1327. The 401 aa residue product is predicted to have a hydrophobic signal peptide of approximately 31 residues at its N-terminus, and 4 potential sites of N-linked  
25 glycosylation. No hydrophobic transmembrane spanning sequence was identified using the PepPlot program (Wisconsin GCG package, version 8.1). The deduced 401 aa sequence was then used to search the protein database. Again, there were no existing matches,  
30 although there appeared to be a strong similarity to many members of the TNFR superfamily, most notably the human and mouse TNFR-II. A sequence alignment of this novel protein with known members of the TNFR-superfamily was prepared using the Pileup program, and  
35 then modified by PrettyPlot (Wisconsin GCG package,

version 8.1). This alignment shows a clear homology between the full length FRI-1 gene product and all other TNFR family members. The homologous region maps to the extracellular domain of TNFR family members, and 5 corresponds to the three or four cysteine-rich repeats found in the ligand binding domain of these proteins. This suggested that the FRI-1 gene encoded a novel TNFR family member. Since no transmembrane spanning region was detected we predicted that this may be a secreted 10 receptor, similar to TNFR-I derived soluble receptors (Kohno *et al.* (1990), *Proc. Natl. Acad. Sci. USA* 87: 8331-5). Due to the apparent biological activity of the FRI-1 gene (*vide infra*), the product was named Osteoprotegerin (OPG).

15

#### EXAMPLE 2

##### OPG mRNA Expression Patterns in Tissues

Multiple human tissue northern blots (Clonetech) were probed with a  $^{32}\text{P}$ -dCTP labelled FRI-1 PCR product to detect the size of the human transcript and to 20 determine patterns of expression. Northern blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS, and 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA for 2-4 hr at 42°C. The blots were then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's 25 solution, 0.1% SDS, 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C. The blots were then washed in 2X SSC for 10 min at room temperature, 1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15 min.

30

Using a probe derived from the rat gene, a predominant mRNA species with a relative molecular mass of about 2.4 kb is detected in several tissues, including kidney, liver, placenta, and heart. Highest levels are detected in the kidney. A large mRNA species 35 of Mr 4.5 and 7.5 kb was detected in skeletal muscle

and pancreas. In human fetal tissue, kidney was found to express relatively high levels of the 2.4 kb mRNA. Using a human probe (vide infra), only the 2.4 kb transcript is detected in these same tissues. In 5 addition, relatively high levels of the 2.4 kb transcript was detected in the lymph node, thymus, spleen and appendix. The size of the transcript detected by both the rat and human Osteoprotegerin gene is almost identical to the length of the rat pB1.1 10 FRI-1 insert, suggesting it was a full length cDNA clone.

**EXAMPLE 3**

**Systemic delivery of OPG in transgenic mice**

The rat OPG clone pB1.1 was used as template to 15 PCR amplify the coding region for subcloning into an ApoE-liver specific expression vector (Simonet *et al.* J. Clin. Invest. **94**, 1310-1319 (1994), and PCT Application No. US94/11675 and co-owned U.S. Serial No. 08/221,767. The following 5' and 3' oligonucleotide 20 primers were used for PCR amplification, respectively:

5'-GACTAGTCCCACAATGAACAAAGTGGCTGTG-3'

(SEQ ID NO:9)

5'-ATAAGAATGCGGCCGCTAAACTATGAAACAGCCCAGTGACCATTC-3'

(SEQ ID NO:10)

25 The PCR reaction mixture (Boehringer-Mannheim) was treated as follows: 94°C for 1 minute, 1 cycle; 94°C for 20 sec, 62°C for 30 sec, and 74 C for 1 minute, 25 cycles. Following amplification, the samples were purified over Qiagen PCR columns and digested overnight 30 with SpeI and NotI restriction enzymes. The digested products were extracted and precipitated and subcloned into the ApoE promoter expression vector. Prior to microinjecting the resulting clone, HE-OPG, it was sequenced to ensure it was mutation-free.

35 The HE-OPG plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified

plasmid DNA was digested with XhoI and Ase I, and the 3.6 kb transgene insert was purified by gel electrophoresis. The purified fragment was diluted to a stock injection solution of 1 µg/ml in 5 mM Tris, pH 5 7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x BDF1-bred mice were injected essentially as described (Brinster *et al.* (1985), Proc. Natl. Acad. Sci. USA 82: 10 4338), except that injection needles were beveled and siliconized before use. Embryos were cultured overnight in a CO<sub>2</sub> incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

Following term pregnancy, 49 offspring were obtained from implantation of microinjected embryos. 15 The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the human Apo E intron which was included in the expression vector. The oligos used for PCR amplification were:

20 5'- GCC TCT AGA AAG AGC TGG GAC-3' (SEQ ID NO:11)  
5'- CGC CGT GTT CCA TTT ATG AGC-3' (SEQ ID NO:12)

The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were 25 identified as PCR positive transgenic founders.

At 8-10 weeks of age, five transgenic founders (2, 11, 16, 17, and 28) and five controls (1, 12, 15, 18, and 30) were sacrificed for necropsy and pathological analysis. Liver was isolated from the remaining 4 30 founders by partial hepatectomy. For partial hepatectomy, the mice were anesthetized and a lobe of liver was surgically removed. Total cellular RNA was isolated from livers of all transgenic founders, and 5 negative control littermates as described (McDonald *et* 35 *al.* Meth. Enzymol. 152, 219 (1987)). Northern blot

analysis was performed on these samples to assess the level of transgene expression. Approximately 10ug of total RNA from each animal liver was resolved by electrophoresis denaturing gels (Ogden *et al.* Meth. Enzymol 152, 61 (1987)), then transferred to HYBOND-N nylon membrane (Amersham), and probed with <sup>32</sup>P dCTP-labelled pB1.1 insert DNA. Hybridization was performed overnight at 42°C in 50% Formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution, 100 µg/ml denatured salmon sperm DNA and 2-4 x 10<sup>6</sup> cpm of labeled probe/ml of hybridization buffer. Following hybridization, blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each, and then twice in 0.1 x SSC, 0.1% SDS at 55°C for 5-10 min each. Expression of the transgene in founder and control littermates was determined following autoradiography.

The northern blot data indicate that 7 of the transgenic founders express detectable levels of the transgene mRNA (animal #'s 2,11,16,17,22,33, and 45). The negative control mice and one of the founders (#28) expressed no transgene-related mRNA. Since OPG is predicted to be a secreted protein, overexpression of transgene mRNA should be a proxy for the level of systemically delivered gene product. Of the PCR and northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

**EXAMPLE 4**

30                   Biological activity of OPG

Five of the transgenic mice (animals 2,11,16,17 and 28) and 5 control littermates (animals 1,12,15,18, and 30) were sacrificed for necropsy and pathological analysis using the following procedures:

Prior to euthanasia, all animals had their identification numbers verified, then were weighed, anesthetized and blood drawn. The blood was saved as both serum and whole blood for a complete serum chemistry and hematology panel. Radiography was performed just after terminal anesthesia by lethal CO<sub>2</sub> inhalation, and prior to the gross dissection.

Following this, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected included the liver, spleen, pancreas, stomach, duodenum, ileum, colon, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, eosphagus, thyroid, jejunem, cecum, rectum, adrenals, urinary bladder, and skeletal muscle. Prior to fixation the whole organ weights were determined for the liver, stomach, kidney, adrenals, spleen, and thymus. After fixation the tissues were processed into paraffin blocks, and 3 um sections were obtained. Bone tissue was decalcified using a formic acid solution, and all sections were stained with hematoxylin and eosin. In addition, staining with Gomori's reticulin and Masson's trichrome were performed on certain tissues. Enzyme histochemistry was performed to determine the expression of tartrate resistant acid phosphatase (TRAP), an enyzme highly expressed by osteoclasts, multinucleated bone-resorbing cells of monocyte-macrophage lineage.

Immunohistochemistry for BrdU and F480 monocyte-macrophage surface antigen was also performed to detect replicating cells and cells of the monocyte-macrophage lineage, respectively. To detect F480 surface antigen expression, formalin fixed, paraffin embedded 4 $\mu$ m sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-

mouse F480 (Harlan, Indianapolis, IN). This antibody was detected by biotinylated rabbit anti-rat immunoglobulins, peroxidase conjugated strepavidin (BioGenex San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with hematoxylin.

Upon gross dissection and observation of visceral tissues, no abnormalities were found in the transgene expressors or control littermates. Analysis of organ weight indicate that spleen size increased by approximately 38% in the transgenic mice relative to controls. There was a slight enlargement of platelet size and increased circulating unstained cells in the transgene expressors. There was a marginal decrease in platelet levels in the transgene expressors. In addition, the serum uric acid, urea nitrogen, and alkaline phosphatase levels all trended lower in the transgene expressors. The expressors were found to have increased radiodensity of the skeleton, including long bones (femurs), vertebrae, and flat bones (pelvis). The relative size of femurs in the expressors were not different from the control mice.

Histological analysis of stained sections of bone from the OPG expressors show severe osteopetrosis with the presence of cartilage remnants from the primary spongiosa seen within bone trabeculae in the diaphysis of the femur. A clearly defined cortex was not identifiable in the sections of femur. In normal animals, the central diaphysis is filled with bone marrow. Sections of vertebra also show osteopetrotic changes implying that the OPG-induced skeletal changes were systemic. The residual bone marrow showed predominantly myeloid elements. Megakaryocytes were present. Reticulin stains showed no evidence for reticulin deposition. Immunohistochemistry for F480, a cell surface antigen expressed by cells of monocyte-

macrophage derivation in the mouse, showed the presence of F480 positive cells in the marrow spaces. Focally, flattened F480 positive cells could be seen directly adjacent to trabecular bone surfaces.

5       The mesenchymal cells lining the bony trabeculae were flattened and appeared inactive. Based on H&E and TRAP stains, osteoclasts were rarely found on the trabecular bone surfaces in the OPG expressors. In contrast, osteoclasts and/or chondroclasts were seen in  
10      the region of the growth plate resorbing cartilage, but their numbers may be reduced compared to controls. Also, osteoclasts were present on the cortical surface of the metaphysis where modelling activity is usually robust. The predominant difference between the  
15      expressors and controls was the profound decrease in trabecular osteoclasts, both in the vertebrae and femurs. The extent of bone accumulation was directly correlated with the level of OPG transgene mRNA detected by northern blotting of total liver RNA.

20       The spleens from the OPG expressors had an increased amount of red pulp with the expansion due to increased hematopoiesis. All hematopoietic lineages are represented. F480 positive cells were present in both control and OPG expressors in the red pulp. Two of the  
25      expressors (2 and 17) had foci of extramedullary hematopoiesis within the liver and this is likely due to the osteopetrotic marrow.

30       There were no observable abnormalities in the thymus, lymph nodes, gastrointestinal tract, pancreato-hepatobiliary tract, respiratory tract, reproductive system, genito-urinary system, skin, nervous system, heart and aorta, breast, skeletal muscle and fat.

**EXAMPLE 5**

**Isolation of mouse and human OPG cDNA**

35       A cDNA clone corresponding to the 5' end of the mouse OPG mRNA was isolated from a mouse kidney cDNA

library (Clontech) by PCR amplification. The oligonucleotides were derived from the rat OPG cDNA sequence and are shown below:

5           5'-ATCAAAGGCAGGGCATACTTCCTG-3' (SEQ ID NO:13)  
5           5'-GTTGCACTCCTGTTCACGGTCTG-3' (SEQ ID NO:14)  
5           5'-CAAGACACCTTGAAGGGCCTGATG-3' (SEQ ID NO:15)  
5           5'-TAACTTTACAGAAGAGCATCAGC-3' (SEQ ID NO:16)

10          5'-AGCGCGGCCGCATGAACAAGTGGCTGTGCTGCG-3' (SEQ ID NO:17)  
10          5'-AGCTCTAGAGAACAGCCCAGTGACCATTCC-3' (SEQ ID NO:18)

The partial and full-length cDNA products obtained in this process were sequenced. The full-length product was digested with Not I and XbaI, then directionally cloned into the plasmid vector pRcCMV (Invitrogen). The 15 resulting plasmid was named pRcCMV-Mu-OPG. The nucleotide sequence of the cloned product was compared to the rat OPG cDNA sequence. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 88% identical. The mouse cDNA 20 sequence contained a 401 aa LORF, which was compared to the rat OPG sequence and found to be about 94% identical without gaps. This indicates that the mouse cDNA sequence isolated encodes the murine OPG, and that the sequence and structure has been highly conserved 25 throughout evolution. The mouse OPG sequence contains an identical putative signal peptide at its N-terminus, and all 4 potential sites of N-linked glycosylation are conserved.

A partial human OPG cDNA was cloned from a human 30 kidney cDNA library using the following rat-specific oligonucleotides:

5'-GTG AAG CTG TGC AAG AAC CTG ATG-3' (SEQ ID NO:19)  
5'-ATC AAA GGC AGG GCA TAC TTC CTG-3' (SEQ ID NO:20)

This PCR product was sequenced and used to design 35 primers for amplifying the 3' end of the human cDNA using a human OPG genomic clone in lambda as template:

5'-TCCGTAAGAACAGCCCAGTGACC-3' (SEQ ID NO:29)

5'-CAGATCCTGAAGCTGCTCAGTTG-3' (SEQ ID NO:21)

The amplified PCR product was sequenced, and together with the 5' end sequence, was used to design 5' and 3' human-specific primers useful for amplifying 5 the entire human OPG cDNA coding sequences:

5'-AGCGCGGCCGCGGGGACCACAAATGAACAAAGTTG-3' (SEQ ID NO:22)

5'-AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3' (SEQ ID NO:23)

The full-length human PCR product was sequenced, then directionally cloned into the plasmid vector 10 pRcCMV (Invitrogen) using Not I and Xba I. The resulting plasmid was named pRcCMV-human OPG. The nucleotide sequence of the cloned product was compared to the rat and mouse OPG cDNA sequences. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA 15 sequences are approximately 78-88% identical to the human OPG cDNA. The human OPG cDNA sequence also contained a 401 aa LORF, and it was compared to the rat and mouse protein sequences. The predicted human OPG is approximately 85% identical, and about 90% identical 20 to the rat and mouse proteins, respectively. Sequence alignment of rat, mouse and human proteins show that they have been highly conserved during evolution. The human protein is predicted to have a N-terminal signal peptide, and 5 potential sites of N-linked 25 glycosylation, 4 of which are conserved between the rat and mouse OPG.

The DNA and predicted amino acid sequence of mouse OPG is shown in Figure 9A and 9B (SEQ ID NO:122). The DNA and predicted amino acid sequence of human OPG is 30 shown in Figure 9C and 9D (SEQ ID NO:124). A comparison of the rat, mouse and human OPG amino acid sequences is shown in Figure 9E and 9F.

Isolation of additional human OPG cDNA clones revealed the presence of a G to C base change at 35 position 103 of the DNA sequence shown in Figure 9C. This nucleotide change results in substitution of an

asparagine for a lysine at position 3 of the amino acid sequence shown in Figure 9C. The remainder of the sequence in clones having this change was identical to that in Figure 9C and 9D.

5

#### EXAMPLE 6

##### OPG three-dimensional structure modelling

The amino-terminal portion of OPG has homology to the extracellular portion of all known members of the TNFR superfamily (Figure 1C). The most notable motif in this region of TNFR-related genes is an about 40 amino acid, cysteine-rich repeat sequence which folds into distinct structures (Banner *et al.* (1993), *Cell* 73: 431-45). This motif is usually displayed in four (range 3-6) tandem repeats (see Figure 1C), and is known to be involved in ligand binding (Beutler and van Huffel (1994), *Science* 264: 667-73). Each repeat usually contains six interspaced cysteine residues, which are involved in forming three intradomain disulfide bonds, termed SS1, SS2, and SS3 (Banner *et al.*, *ibid*). In some receptors, such as TNFR2, CD30 and CD40, some of the repeat domains contain only two intrachain disulfide bonds (SS1 and SS3).

The human OPG sequence was aligned to a TNFR1 extracellular domain profile using methods described by Luethy, *et al.*, *ibid*, and the results were graphically displayed using the PrettyPlot program from the Wisconsin Package, version 8.1 (Genetics Computer Group, Madison, WI) (Figure 10). The alignment indicates a clear conservation of cysteine residues involved in formation of domains 1-4. This alignment was then used to construct a three-dimensional (3-D) model of the human OPG N-terminal domain using the known 3-D structure of the extracellular domain of p55 TNFR1 (Banner *et al.*, *ibid*) as the template. To do this the atomic coordinates of the peptide backbone and side chains of identical residues were copied from the

crystal structure coordinates of TNFR1. Following this, the remaining coordinates for the insertions and different side chains were generated using the LOOK program (Molecular Applications Group, Palo Alto, CA).  
5 The 3-D model was then refined by minimizing its conformational energy using LOOK.

By analogy with other TNFR family members, it is assumed that OPG binds to a ligand. For the purpose of modelling the interaction of OPG with its ligand, the  
10 crystal structure of TNF- $\beta$  was used to simulate a 3-D representation of an "OPG ligand". This data was graphically displayed (see Figure 11) using Molscript (Kraulis (1991), J. Appl. Cryst. 24: 946-50). A model for the OPG/ligand complex with 3 TNF $\beta$  and 3 OPG  
15 molecules was constructed where the relative positions of OPG are identical to TNFR1 in the crystal structure. This model was then used to find the residues of OPG that could interact with its ligand using the following approach: The solvent accessible area of all residues  
20 in the complex and one single OPG model were calculated. The residues that have different accessibility in the complex than in the monomer are likely to interact with the ligand.

The human and mouse OPG amino acid sequences were  
25 realigned using this information to highlight sequences comprising each of the cysteine rich domains 1-4 (Figure 12A and 12B). Each domain has individual structural characteristics which can be predicted.

Domain 1: Contains 4 cysteines involved in SS2 (C41 to C54) and SS3 (C44 to C62) disulfide bonds. Although no SS1 bond is evident based on disulfide bridges, the conserved tyrosine at position 28 is homologous to Y20 in TNFR1, which is known to be involved in interacting with H66 to aid in domain formation. OPG has a homologous histidine at position  
35

75, suggesting OPG Y28 and H75 stack together in the native protein, as do the homologous residues in TNFR1. Therefore, both of these residues may indeed be important for biological activity, and N-terminal OPG 5 truncations up to and beyond Y28 may have altered activity. In addition, residues E34 and K43 are predicted to interact with a bound ligand based on our 3-dimensional model.

10        Domain 2: Contains six cysteines and is predicted to contain SS1 (C65 to C80), SS2 (C83 to C98) and SS3 (C87 to C105) disulfide bonds. This region of OPG also contains an region stretching from P66-Q91 which aligns to the portion of TNFR1 domain 2 which forms close contacts with TNF $\beta$  (see above), and may interact with 15 an OPG ligand. In particular residues P66, H68, Y69, Y70, T71, D72, S73, H75, T76, S77, D78, E79, L81, Y82, P85, V86, K88, E89, L90, and Q91 are predicted to interact with a bound ligand based on our structural data.

20        Domain 3: Contains 4 cysteines involved in SS1 (C107 to C 118) and SS3 (C124 to C142) disulfide bonds, but not an SS2 bond. Based on our structural data, residues E115, L118 and K119 are predicted in to interact with an OPG ligand.

25        Domain 4: Contains 4 cysteines involved in SS1 (C145 to C160) and SS3 (C166 to C185) disulfide bonds, but not an SS2 bond, similar to domain 3. Our structural data predict that E153 and S155 interact with an OPG ligand.

30        Thus, the predicted structural model for OPG identifies a number of highly conserved residues which are likely to be important for its biological activity.

**EXAMPLE 7**

Production of recombinant secreted

OPG in mammalian cells

35

To determine if OPG is actually a secreted protein, mouse OPG cDNA was fused to the human IgG1 Fc domain as a tag (Capon *et al.* *Nature* 337, 525-531 (1989)), and expressed in human 293 fibroblasts. Fc fusions were carried out using the vector pFc-A3. pFc-A3 contains the region encoding the Fc portion of human immunoglobulin IgG- $\gamma$ 1 heavy chain (Ellison *et al.* *ibid*) from the first amino acid of the hinge domain (Glu-99) to the carboxyl terminus and is flanked by a 5'-NotI fusion site and 3'-SalI and XbaI sites. The plasmid was constructed by PCR amplification of the human spleen cDNA library (Clontech). PCR reactions were in a final volume of 100  $\mu$ l and employed 2 units of Vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10  $\mu$ M  $(\text{NH}_4)_2\text{SO}_4$ , 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100 with 400  $\mu$ M each dNTP and 1 ng of the cDNA library to be amplified together with 1  $\mu$ M of each primer. Reactions were initiated by denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 73°C for 2 min. The 5' primer  
5' ATAGCGGCCGCTGAGCCCAAATCTTGTGACAAAACTCAC 3'  
(SEQ ID NO:24)  
incorporated a NotI site immediately 5' to the first residue (Glu-99) of the hinge domain of IgG- $\gamma$ 1. The 3' primer  
5' -TCTAGAGTCGACTTATCATTACCCGGAGACAGGGAGAGGCTT-3'  
(SEQ ID NO:25)  
incorporated SalI and XbaI sites. The 717-bp PCR product was digested with NotI and SalI, isolated by electrophoresis through 1% agarose (FMC Corp.), purified by the Geneclean procedure (BIO 101, Inc.) and cloned into NotI, SalI-digested pBluescript II KS vector (Stratagene). The insert in the resulting plasmid, pFc-A3, was sequenced to confirm the fidelity of the PCR reaction.

The cloned mouse cDNA in plasmid pRcCMV-MuOPG was amplified using the following two sets of primer pairs:

Pair 1:

5 5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAAG-3'  
(SEQ ID NO:26)  
5'-CCTCTGCGGCCGCTAACGAGCTTATTTCACGGATTGAACCTG-3'  
(SEQ ID NO:27)

Pair 2:

10 5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAAG-3'  
(SEQ ID NO:28)  
5'-CCTCTGCGGCCGCTGTTGCATTCCTTTCTG-3'  
(SEQ ID NO:30)

The first pair amplifies the entire OPG LORF, and creates a NotI restriction site which is compatible 15 with the in-frame Not I site in Fc fusion vector pFcA3. pFcA3 was prepared by engineering a NotI restriction site 5' to aspartic acid residue 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the 20 junction between the OPG and IgG Fc region. This product, when linked to the Fc portion, would encode all 401 OPG residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (F1.Fc). The second primer pair amplifies the DNA sequences encoding 25 the first 180 amino acid residues of OPG, which encompasses its putative ligand binding domain. As above, the 3' primer creates an artificial Not I restriction site which fuses the C-terminal truncated OPG LORF at position threonine 180 directly to the IgG1 30 Fc domain (CT.fc).

The amino acid sequence junction linking OPG residue 401 and aseptic acid residue 221 of the human Fc region can be modified as follows: The DNA encoding residues 216-220 of the human Fc region can be deleted 35 as described below, or the cysteine residue corresponding to C220 of the human Fc region can be

5 mutated to either serine or alanine. OPF-Fc fusion protein encoded by these modified vectors can be transfected into human 293 cells, or CHO cells, and recombinant OPG-Fc fusion protein purified as described below.

Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is capable of episomal replication in 293-EBNA-1 cells.

10 The parent pCEP4, and pCEP4-F1.Fc and pCEP4-CT.Fc vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 µg/ml hygromycin to select for vector expression, and the resulting drug-

15 resistant mass cultures were grown to confluence. The cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced

20 by the drug resistant 293 cultures. In the pCEP4-F1.FC and the pCEP4-CT.FC conditioned media, unique bands of the predicted sizes were abundantly secreted (see Figures 13B and 13C). The full-length Fc fusion protein accumulated to a high concentration, indicating that it

25 may be stable. Both Fc fusion proteins were detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant OPG products.

The full length OPG-Fc fusion protein was purified

30 by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira *et al.* (*J. Biol. Chem.* 262, 10-35 (1987)).

The following amino acid sequence was read after 19 cycles:

NH<sub>2</sub>-E T L P P K Y L H Y D P E T G H Q L L-CO<sub>2</sub>H  
(SEQ ID NO:31)

5 This sequence was identical to the predicted mouse OPG amino acid sequence beginning at amino acid residue 22, suggesting that the natural mammalian leader cleavage site is between amino acid residues Q21-E22, not between Y31-D32 as originally predicted. The 10 expression experiments performed in 293-EBNA cells with pCEP4-F1.Fc and pCEP4-CT.Fc demonstrate that OPG is a secreted protein, and may act systemically to bind its ligand.

15 Procedures similar to those used to construct and express the muOPG[22-180]-Fc and muOPG[22-401]-Fc fusions were employed for additional mouse and human OPG-Fc fusion proteins.

20 Murine OPG cDNA encoding amino acids 1-185 fused to the Fc region of human IgG1 [muOPG Ct(185).Fc] was constructed as follows. Murine OPG cDNA from plasmid pRcCMV Mu Osteoprotegerin (described in Example 5) was amplified using the following primer pair in a polymerase chain reaction as described above:

1333-82:

25 5'-TCC CTT GCC CTG ACC ACT CTT-3'  
(SEQ ID NO:32)

1333-80:

5'-CCT CTG CGG CCG CAC ACA CGT TGT CAT GTG TTG C-3'  
(SEQ ID NO:33)

30 This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 63-185 (corresponding to bp 278-645) of the OPG reading frame as shown in Figure 9A. The 3' primer contains a Not I restriction site which is compatible with the in-frame 35 Not I site of the Fc fusion vector pFcA3. The product also spans a unique EcoRI restriction site located at

bp 436. The amplified PCR product was purified, cleaved with NotI and EcoRI, and the resulting EcoRI-NotI restriction fragment was purified. The vector pCEP4 having the murine 1-401 OPG-Fc fusion insert was 5 cleaved with EcoRI and NotI, purified, and ligated to the PCR product generated above. The resulting pCEP4-based expression vector encodes OPG residues 1-185 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-185.Fc fusion 10 vector was transfected into 293 cells, drug selected, and conditioned media was produced as described above. The resulting secreted murine OPG 1-185.Fc fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended 15 procedures.

Murine OPG DNA encoding amino acid residues 1-194 fused to the Fc region of human IgG1 (muOPG Ct(194).Fc) was constructed as follows. Mouse OPG cDNA from plasmid pRcCMV Mu-Osteoprotegerin was amplified using the 20 following primer pairs:

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3'  
(SEQ ID NO:34)

1333-81:

25 5'-CCT CTG CGG CCG CCT TTT GCG TGG CTT CTC TGT T-3'  
(SEQ ID NO:35)

This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 70-194 (corresponding to bp 298-672) of the OPG reading frame. 30 The 3' primer contains a Not I restriction site which is compatible with the in-frame Not I site of the Fc fusion vector pFca3. The product also spans a unique EcoRI restriction site located at bp 436. The amplified PCR product was cloned into the murine OPG[1-401] Fc 35 fusion vector as described above. The resulting pCEP4-based expression vector encodes OPG residues 1-194

directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-194.Fc fusion vector was transfected into 293 cells, drug selected, and conditioned media was produced. The resulting 5 secreted fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acids 1-401 fused to the Fc region of human IgG1 was constructed as follows. 10 Human OPG DNA in plasmid pRcCMV-hu osteoprotegerin (described in Example 5) was amplified using the following oligonucleotide primers:

1254-90:

5'CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'

15 (SEQ ID NO:36)

1254-95:

5'-CCT CTG CGG CCG CTA AGC AGC TTA TTT TTA CTG AAT GG-3'

(SEQ ID NO:37)

The resulting PCR product encodes the full-length 20 human OPG and creates a Not I restriction site which is compatible with the in-frame Not I site Fc fusion vector FcA3. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above. The resulting expression vector encodes human OPG residues 25 1-401 directly followed by 227 amino acid residues of the human IgG1 Fc region. Conditioned media from transfected and drug selected cells was produced and the huOPG F1.Fc fusion product was purified by Protein-A column chromatography (Pierce) using the 30 manufacturers recommended procedures.

Human OPG DNA encoding amino acid residues 1-201 fused to the Fc region of human IgG1 [huOPG Ct(201).Fc] was constructed as follows. The cloned human OPG cDNA from plasmid pRrCMV-hu osteoprotegerin was amplified by 35 PCR using the following oligonucleotide primer pair:

1254-90:

5'-CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'  
(SEQ ID NO:38)

1254-92:

5' -CCT CTG CGG CCG CCA GGG TAA CAT CTA TTC CAC-3'  
5 (SEQ ID NO:39)

This primer pair amplifies the human OPG cDNA region encoding amino acid residues 1-201 of the OPG reading frame, and creates a Not I restriction site at the 3' end which is compatible with the in-frame Not I site Fc fusion vector FcA3. This product, when linked to the Fc portion, encodes OPG residues 1-201 directly followed by all 221 amino acid residues of the human IgG1 Fc region. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above. Conditioned media from transfected and drug selected cells was produced, and the hu OPG Ct(201).Fc fusion products purified by Protein-A column chromatography (Pierce) using the manufacturer's recommended procedures.

20 The following procedures were used to construct and express unfused mouse and human OPG.

A plasmid for mammalian expression of full-length murine OPG (residues 1-401) was generated by PCR amplification of the murine OPG cDNA insert from pRcCMV Mu-Osteoprotegerin and subcloned into the expression vector pDSR $\alpha$  (DeClerck et. al. J. Biol. Chem. 266, 3893 (1991)). The following oligonucleotide primers were used:

1295-26:

30 5'-CCG AAG CTT CCA CCA TGA ACA AGT GGC TGT GCT GC-3'  
(SEQ ID NO:40)

1295-27:

5'-CCT CTG TCG ACT ATT ATA AGC AGC TTA TTT TCA CGG ATT G-3'  
(SEQ ID NO:41)

35 The murine OPG full length reading frame was amplified by PCR as described above. The PCR product

was purified and digested with restriction endonucleases Hind III and XbaI (Boehringer Mannheim, Indianapolis, IN) under the manufacturers recommended conditions, then ligated to Hind III and Xba I digested pDSR $\alpha$ . Recombinant clones were detected by restriction endonuclease digestion, then sequenced to ensure no mutations were produced during the PCR amplification steps.

The resulting plasmid, pDSR $\alpha$ -muOPG was introduced into Chinese hamster ovary (CHO) cells by calcium mediated transfection (Wigler *et al.* (1977), *Cell* 11: 233). Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the murine OPG recombinant protein was monitored by western blot analysis of CHO cell conditioned media. High expressing cells were selected, and OPG expression was further amplified by treatment with methotrexate as described (DeClerck *et al.*, *ibid.*). Conditioned media from CHO cell lines was produced for further purification of recombinant secreted murine OPG.

A plasmid for mammalian expression of full-length human OPG (amino acids 1-401) was generated by subcloning the cDNA insert in pRcCMV-hu Osteoprotegerin directly into vector pDSR $\alpha$  (DeClerck *et al.*, *ibid.*). The pRcCMV-OPG plasmid was digested to completion with Not I, blunt ended with Klenow, then digested to completion with XbaI. Vector DNA was digested with HindIII, blunt ended with Klenow, then digested with XbaI, then ligated to the OPG insert. Recombinant plasmids were then sequenced to confirm proper orientation of the human OPG cDNA.

The resulting plasmid pDSR $\alpha$ -huOPG was introduced into Chinese hamster ovary (CHO) cells as described

above. Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the human OPG recombinant protein was 5 monitored by western blot analysis of CHO cell conditioned media. High expressing clones were selected, and OPG expression was further amplified by treatment with methotrexate. Conditioned media from CHO cell lines expressing human OPG was produced for 10 protein purification.

Expression vectors for murine OPG encoding residues 1-185 were constructed as follows. Murine OPG cDNA from pRccMV-Mu OPG was amplified using the following oligonucleotide primers:

15 1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:42)

1356-12:

5'-CCT CTG TCG ACT TAA CAC ACG TTG TCA TGT GTT GC-3'  
(SEQ ID NO:43)

20 This primer pair amplifies the murine OPG cDNA region encoding amino acids 63-185 of the OPG reading frame (bp 278-645) and contains an artificial stop codon directly after the cysteine codon (C185), which is followed by an artificial Sal I restriction 25 endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large 30 fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel 35 purified. This fragment, which contains an open reading frame encoding residues 1-185 was then subcloned into a

Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a cysteine residue located at position 185. Conditioned media from transfected and drug selected cells was produced as described above.

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:44)

1356-13:

10 5'-CCT CTG TCG ACT TAC TTT TGC GTG GCT TCT CTG TT-3'  
(SEQ ID NO:45)

This primer pair amplifies the murine OPG cDNA region encoding amino acids 70-194 of the OPG reading frame (bp 298-672) and contains an artificial stop codon directly after the lysine codon (K194), which is followed by an artificial Sal I restriction endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel purified. This fragment, which contains a open reading frame encoding residues 1-185 was then subcloned into a Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a lysine at position 194. Conditioned media from transfected and drug selected cells was produced as described above.

Several mutations were generated at the 5' end of the huOPG [22-401]-Fc gene that introduce either amino acid substitutions, or deletions, of OPG between

residues 22 through 32. All mutations were generated with the "QuickChange™ Site-Directed Mutagenesis Kit" (Stratagene, San Diego, CA) using the manufacturer's recommended conditions. Briefly, reaction mix 5 containing huOPG [22-401]-Fc plasmid DNA template and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent *E. coli* 10 XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to delete residues 22-26 of the human OPG gene, resulting in the production of a huOPG [27-401]-Fc fusion protein:

15 1436-11:

5'-TGG ACC ACC CAG AAG TAC CTT CAT TAT GAC-3' (SEQ ID NO:140)

1436-12:

5'-GTC ATA ATG AAG GTA CTT CTG GGT CCA-3' (SEQ ID NO:141)

The following primer pairs were used to delete 20 residues 22-28 of the human OPG gene, resulting in the production of a huOPG [29-401]-Fc fusion protein:

1436-17:

5'-GGA CCA CCC AGC TTC ATT ATG ACG AAG AAA C-3' (SEQ ID NO:142)

1436-18:

25 5'-GTT TCT TCG TCA TAA TGA AGC TGG GTG GTC C-3' (SEQ ID NO:143)

The following primer pairs were used to delete residues 22-31 of the human OPG gene, resulting in the production of a huOPG [32-401]-Fc fusion protein:

1436-27:

30 5'-GTG GAC CAC CCA GGA CGA AGA AAC CTC TC-3' (SEQ ID NO:144)

1436-28:

5'-GAG AGG TTT CTT CGT CCT GGG TGG TCC AC-3' (SEQ ID NO:145)

The following primer pairs were used to change the codon for tyrosine residue 28 to phenylalanine of the 35 human OPG gene, resulting in the production of a huOPG [22-401]-Fc Y28F fusion protein:

1436-29:

5'-CGT TTC CTC CAA AGT TCC TTC ATT ATG AC-3' (SEQ ID NO:146)

1436-30:

5'-GTC ATA ATG AAG GAA CTT TGG AGG AAA CG-3' (SEQ ID NO:147)

5 The following primer pairs were used to change the codon for proline residue 26 to alanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc P26A fusion protein:

1429-83:

10 5'-GGA AAC GTT TCC TGC AAA GTA CCT TCA TTA TG-3 (SEQ ID NO:148)

1429-84:

5'-CAT AAT GAA GGT ACT TTG CAG GAA ACG TTT CC-3' (SEQ ID NO:149)

Each resulting muOPG [22-401]-Fc plasmid containing the appropriate mutation was then 15 transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in Example 11.

20 **EXAMPLE 8**

Expression of OPG in E. coli

A. Bacterial Expression Vectors

pAMG21

The expression plasmid pAMG21 can be derived from 25 the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be derived from the Amgen expression vector system described in US Patent No. 4,710,473. The pCFM1656 plasmid can be derived from the described pCFM836 plasmid (Patent No. 4,710,473) by: (a) 30 destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end ligation; (b) replacing the DNA sequence between the unique AatII and ClaI restriction sites containing the synthetic P<sub>L</sub> promoter with a similar fragment 35 obtained from pCFM636 (patent No. 4,710,473) containing the PL promoter

**AatII**

5' CTAATTCCGCTCTCACCTACCAAAACAATGCCCTGGCAAAAAATAATTAT-  
 3' TGCAGATTAAGGCGAGAGTGGATGGTTACGGGGGACGTTTTATTAAGTATA-  
  
 5 -AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-  
 -TTTTTGATGTCTATTGGTAGACGCCACTATTAATAGAGACCGCCACAACGTATT-  
  
 -TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)  
 -ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

10 **ClaI**

and then (c) substituting the small DNA sequence between the unique ClaI and KpnI restriction sites with the following oligonucleotide:

5' CGATTGATTCTAGAAGGAGGAATAACATATGGTTAACCGTTGGAATTGGTAC3'  
 15 (SEQ ID NO:48)  
 3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGAACCTTAAGC 5'  
 (SEQ ID NO:49)

## Clal KpnI

The expression plasmid pAMG21 can then be derived from pCFM1656 by making a series of site directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the *Bgl*II site (plasmid bp # 180) immediately 5' to the plasmid replication promoter *PcopB* and proceeding toward the plasmid replication genes, the base pair changes are as follows:

Table 4

<u>pAMG21 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG21</u>
30	# 204	T/A
	# 428	A/T
	# 509	G/C
	# 617	- -
	# 679	G/C
35	# 980	T/A
	# 994	G/C
	# 1004	A/T
	# 1007	C/G
	# 1028	A/T
40	# 1047	C/G
	# 1178	G/C
	# 1466	G/C
	# 2028	G/C
	# 2187	C/G
		insert two G/C bp
		T/A
		C/G
		A/T
		C/G
		T/A
		bp deletion
		T/A

	# 2480	A/T	T/A
	# 2499-2502	AGTG TCAC	GTCA CAGT
5	# 2642	TCCGAGC AGGCTCG	7 bp deletion
10	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[AatII sticky end] 5' GCGTAACGTATGCATGGTCTCC-  
(position #4358 in pAMG21) 3' TGCACGCATTGCATACTGACCGAGGG-

20 -CCATGCGAGAGTAGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT-  
-GGTACGCTCTCATCCCTGACGGTCCGTAGTTATTTGCTTCCGAGTCAGCTTCTGA-

25 -GGGCCTTCGTTTATCTGTTGTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGC-  
-CCCGGAAAGCAAATAGACAACAAACAGCCACTTGCAGAGGACTCATCCTGTTAGGCG-

30 -CGGGAGCGGATTGAACGTTGCGAACGCAACGGCCGGAGGGTGGCGGGCAGGACGCCGC-  
-GCCCTCGCCTAAACTTGCAACGCTTCGTTGCCGGGCTCCCACCGCCGCTGCCGCG-

35 -CATAAAACTGCCAGGCATCAAATTAAGCAGAACGGCCATCCTGACGGATGCCCTTTGCGT-  
-GTATTTGACGGTCCGTAGTTAATTGCTCTCCGGTAGGACTGCCTACCGAAAAACGCA-

AatII

40 -TTCTACAAACTCTTTGTTATTTCTAAATACATTCAAATATGGACGTCGTACTTAAC-  
-AAGATGTTGAGAAAACAAATAAAAGATTATGTAAGTTATACCTGCAGCATGAATTG-

45 -TTTAAAGTATGGCAATCAATTGCTCTGTTAAATTGCTTAGAAATACTTGGCAGC-  
-AAAATTTCATACCCGTTAGTTAACGAGGACAATTAAACGAAATCTTATGAAACCCTCG-

50 -GGTTTGTGATTGAGTTTCATTGCGCATTGGTAAATGGAAAGTGACCGTGCCTTAC-  
-CCAAACACATAACTCAAAGTAAACGCGTAACCAATTACCTTCACTGGCACGCCAATG-

55 -TACAGCCTAATATTTGAAATATCCCAAGAGCTTTCTCGCATGCCACGCTAAC-  
-ATGTCGGATTATAAAACTTATAGGGTCTCGAAAAGGAAGCGTACGGGTGCGATTG-

60 -ATTCTTTCTCTTTGGTTAAATCGTTGTTGATTATTATTTGCTATATTATTTTC-  
-TAAGAAAAGAGAAAACCAATTAGCAACAAACTAAATAAACGATATAAATAAAAG-

-GATAATTATCAACTAGAGAAGGAACAATTAAATGGTATGTCATACACGCGATGAAAAATA-  
-CTATTAATAGTTGATCTCTGTTAAATTACCATACAAGTATGTCGCTACATTTCAT-

65 -AACTATCTATATAGTTGCTCTCTGAATGTGAAAACCTAACGCGATTCCGAAGCCATTAT-  
-TTGATAGATATCAACAGAAAGAGACTTACACGTTGATTGTAAGGCTTCGGTAATA-

70 -TAGCAGTATGAATAGGGAAACTAAACCCAGTGATAAGACCTGATGATTGCTTCTTTAA-  
-ATCGTCATACTTATCCCTTGATTGGGTCACTATTCTGGACTACTAAAGCGAAGAAATT-

75 -TTACATTGGAGATTTTTATTACAGCATTGTTCAAATATATTCCAATTATCGGTG-  
-AATGTAACCTCTAAAAATAATGTCGAACAAAAGTTATATAAGGTTAATTAGCCAC-

80 -AATGATTGGAGTTAGAATAATCTACTATAGGATCATATTATTAAATTAGCGTCATCAT-

-TTACTAACCTCAATCTTATTAGATGATATCCTAGTATAAAATAATTAAATCGCAGTAGTA-  
 -AATATTGCCTCCATTTCAGGGTAATTATCCAGAATTGAAATATCAGATTAAACCATAAG-  
 -TTATAACGGAGGTAAAAATCCCATTAAATAGGTCTTAACTTATAGTCTAAATTGGTATC-  
 5 -AATGAGGATAAAATGATCGCGAGTAAATAATATTACAATGTACCATTAGTCATATCAG-  
 -TTACTCCTATTACTAGCGCTCATTTATTATAAGTGTACATGGTAAAATCAGTATAGTC-  
 -ATAAGCATTGATTAATATCATATTGCTCTACAGGTTAATTTATTAAATTATTCTGT-  
 10 -TATTCGTAACTAATTATAGTAATAACGAAGATGTCCGAAATTAAATTAAAGACA-  
 -AAGTGTGTCGGCATTATGCTTACCCATCTTTATCCTTACCTATTGTTGTC-  
 -TTCACAGCAGCCGTAACAGAAAGTATGGTAGAGAAATAGGAATGGATAACAAACAG-  
 15 -GCAAGTTTGCCTGTTATATCATTAACCGTAATAGATTGACATTGATTCTAATAA-  
 -CGTTCAAAACGCACAATATATAGTAATTGCCCCATTATCTAACTGTAAACTAAGATTATT-  
 -ATTGGATTTGTCACACTATTATATCGCTGAAATACAATTGTTAACATAAGTACCTG-  
 -TAACCTAAAAACAGTGTGATAATATAGCGAACCTTATGTTAACAAATTGTATTGAC-  
 20 -TAGGATCGTACAGGTTACGCAAGAAAATGGTTGTTAGTCGATTAATCGATTGATT-  
 -ATCCTAGCATGTCCAAATGCCTTACCAACAAATCAGCTAATTAGCTAAACTAA-  
 -CTAGATTTGTTTAACTAATTAAAGGAGGAATAACATATGGTTAACCGCTTGGAAATCGA-  
 25 -GATCTAAACAAAATTGATTAATTCCCTCTTATTGTATACCAATTGCGAACCTTAAGCT-  
  
 SacII  
 -GCTCACTAGTGTGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGGAAAGAA-  
 -CGAGTGATCACAGCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCTTCTT-  
 30 -GAAGAAGAAGAAGAAAGCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA-  
 -CTTCTTCTTCTTCTGGGCTTCGACTCAACCGACGACGGTGGCGACTCGTTAT-  
 -ACTAGCATAACCCCTTGGGCCTCTAACGGGTCTGAGGGGTTTTGCTGAAAGGAGG-  
 35 -TGATCGTATTGGGAACCCCGAGATTGCCAGAACTCCCCAAAAACGACTTCCCTC-  
 -AACCGCTCTTCACGCTTACGC 3' [SacII sticky end] (SEQ ID NO:50)  
 -TTGGCGAGAAGTGGCAGAAGTG 5' (position #5904 in pAMG21)  
 (SEQ ID NO:46)

40 During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

45 pAMG22-His

The expression plasmid pAMG22-His can be derived from the Amgen expression vector pAMG22 by substituting the small DNA sequence between the unique NdeI (#4795) and EcoRI (#4818) restriction sites of pAMG22 with the 50 following oligonucleotide duplex:

NdeI                  NheI                  EcoRI  
 5' TATGAAACATCATCACCACCATGCTAGCGTTAACCGCTTGGG 3'

(SEQ ID NO:51)

3' ACTTTGTAGTAGTGGTAGTGGTAGTACGATCGCAATTGCGAACCTTAA 5'

(SEQ ID NO:52)

MetLysHisHisHisHisHisHisAlaSerValAsnAlaLeuGlu

(SEQ ID NO:168)

pAMG22

5       The expression plasmid pAMG22 can be derived from  
the Amgen expression vector pCFM1656 (ATCC #69576)  
which in turn be derived from the Amgen expression  
vector system described in US Patent No. 4,710,473  
granted December 1, 1987. The pCFM1656 plasmid can be  
10      derived from the described pCFM836 plasmid (Patent No.  
4,710,473) by: (a) destroying the two endogenous NdeI  
restriction sites by end filling with T4 polymerase  
enzyme followed by blunt end ligation; (b) replacing  
the DNA sequence between the unique AatII and ClaI  
15      restriction sites containing the synthetic PL promoter  
with a similar fragment obtained from pCFM636 (patent  
No. 4,710,473) containing the PL promoter

AatII

5'       CTAATTCCGCTCTCACCTACCAAACAATGCCCTGCAAAAAAATAATTCAATAT-  
20      3' TGCAGATTAAGGCGAGAGTGGATGGTTGTTACGGGGGGACGTTTTATTAAAGTATA-

-AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-  
-TTTTTGATGTCATTGGTAGACGCCACTATTTAATAGAGACCGCCACAAGTGTATTT-

25      -TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)  
-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

ClaI

and then (c) substituting the small DNA sequence  
between the unique ClaI and KpnI restriction sites with  
30      the following oligonucleotide:

5' CGATTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTGGTAC 3'  
(SEQ ID NO:55)

3' TAAACTAAGATCTCCTCCTTATTGTATACCAATTGCGAACCTTAAGC 5'  
(SEQ ID NO:56)

35      ClaI

KpnI

The expression plasmid pAMG22 can then be derived from pCFM1656 by making a series of site directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the BglII site (plasmid bp # 180) immediately 5' to the plasmid replication promoter PcopB and proceeding toward the plasmid replication genes, the base pair changes are as follows:

Table 5

	<u>pAMG22 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG22</u>
10	# 204	T/A	C/G
	# 428	A/T	G/C
15	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
20	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
25	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
30	# 2499-2502	AGTG TCAC	GTCA CAGT
	# 2642	TCCGAGC AGGCTCG	7 bp deletion
35	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

40 The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[AatII sticky end] (position #4358 in pAMG22)

5' GCGTAACGTATGCATGGTCTCCCCATGCGAGAGTAGGAACTGCCAGGCATCAA-  
 3' TGCACCGATTGCATACTGACGAGGGTAGCCTCATCCCTGACGGTCCGTAGTT-

5 -ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTCGTTATCTGTTGTTGTCGGT-  
 -TATTTGCTTCCGAGTCAGCTTCTGACCCGGAAAGCAAAATAGACAACAAACAGCCAC-

10 -AACGCTCTCCTGAGTAGGACAAATCCGCCGGAGCGGGATTGAACGTTGCGAAGCAACGG-  
 -TTGCGAGAGGACTCATCCTGTTAGGCGCCCTGCCCTAAACTGCAACGCTTCGTTGCC-

15 -CCCGGAGGGTGGCGGGCAGGACGCCGCATAAACTGCCAGGCATCAAATTAAGCAGAAG-  
 -GGGCCTCCCACCGCCGCTCGGGCGTATTGACGGTCCGTAGTTAATTGCTTC-

20 -GCCATCCTGACGGATGGCCTTTGCGTTCTACAAACTCTTTGTTATTTCTAAAT-  
 -CGGTAGGACTGCCTACCGGAAAAACGAAAGATGTTGAGAAAACAATAAAAGATTAA-

AatII

-ACATTCAAATATGGACGTCTCATATTAAAAAATTCAATTGACAAATGCTAAAATTC-  
 -TGTAAGTTATACCTGCAGAGTATTAAAAATTGTTAAGTAAACTGTTACGATTTAAG-

25 -TTGATTAATATTCTCAATTGTGAGCGCTCACAAATTATCGATTGATTCTAGATTGTT-  
 -AACTAATTATAAGAGTTAACACTCGCAGTGTAAAGCTAAACTAAGATCTAAACTCA-

-TAACTAATTAAAGGAGGAATAACATATGGTAACCGCGTTGGAATTGAGCTCACTAGTGT-  
 -ATTGATTAATTCCCTTATTGTATACCAATTGCGAACCTTAAGCTCGAGTGATCACA-

SacII

-CGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCCGGAAAGAAGAAGAAGAA-  
 -GCTGGACGTCCCATGGTACCTCGAATGAGCTCCTAGGCCTTCTTCTTCTTCTT

30 -GAAAGCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC-  
 -CTTCGGCTTCCTCGACTCAACCGACGACGGTGGCAGTCGTTATTGATCGTATTGG-

35 -CCTTGGGCCTCTAAACGGGCTTGTGAGGGGTTTTTGCTGAAAGGAGGAACCGCTCTCA-  
 -GGAACCCCGGAGATTGCCAGAACTCCCCAAAAACGACTTCCTGGCGAGAAGT-

-CGCTTTCACGC 3' (SEQ ID NO:58)  
 -GCGAGAAGTG 5' (SEQ ID NO:57)

40 [SacII sticky end] (position #5024 in pAMG22)  
 During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

45 B. Human OPG Met[32-401]

In the example, the expression vector used was pAMG21, a derivative of pCFM1656 (ATCC accession no. 69576) which contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter. 50 (See U.S. Patent No. 5,169,318 for description of the lux expression system). The host cell used was GM120 (ATCC accession no. 55764). This host has the lacIQ promoter and lacI gene integrated into a second site in

the host chromosome of a prototrophic E. coli K12 host. Other commonly used E. coli expression vectors and host cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human OPG polypeptide was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as a template plasmid pRcCMV-Hu OPG DNA containing the human OPG cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for 30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22 were phosphorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and Kpnl sticky ends. The phosphorylated linker duplex formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and Kpnl cohesive ends (see Figure 14A) and the Kpnl and BamHI digested and purified PCR product generated using oligo primers #1257-20 and #1257-19 (see above) was directionally inserted between two sites of the plasmid vector pAMG21, namely the NdeI site and BamHI site, using standard recombinant DNA methodology (see Figure 14A and sequences below). The synthetic linker utilized E. coli codons and provided for a N-terminal methionine.

Two clones were selected and plasmid DNA isolated, and the human OPG insert was subsequently DNA sequence confirmed. The resulting pAMG21 plasmid containing

amino acids 32-401 of the human OPG polypeptide immediately preceded in frame by a methionine is referred to as pAMG21-huOPG met[32-401] or pAMG21-huOPG met[32-401].

5           Oligo#1257-19:

5'-TACGCACGGATCCTTATAAGCAGCTTATTTTACTGATTGGAC-3'  
(SEQ ID NO:59)

              Oligo#1257-20:

5'-GTCCTCCTGGTACCTACCTAAAACAAC-3' (SEQ ID NO:60)

10          Oligo#1257-21:

5'-TATGGATGAAGAAACTTCTCATCAGCTGCTGTGATAAATGTCCGCCGGTAC -3'  
(SEQ ID NO:61)

              Oligo#1257-22:

5'-CCGGCGGACATTATCACACAGCAGCTGATGAGAAGTTCTTCATCCA-3'  
15       (SEQ ID NO:47)

              Cultures of pAMG21-huOPG met[32-401] in E. coli GM120 in 2XYT media containing 20 µg/ml kanamycin were incubated at 30°C prior to induction. Induction of huOPG met[32-401] gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and cultures were incubated at either 30°C or 37°C for a further 6 hours. After 6 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies and were then pelleted by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant huOPG met[32-401] gene product was produced insolubly in E. coli.

              Some bacterial pellets were resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemlli sample buffer to 1X final, and β-mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE. A substantially more intense coomassie stained band of approximately 42kDa was observed on a

SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture (Figure 14B).  
The expected gene product would be 370 amino acids in  
5 length and have an expected molecular weight of about 42.2 kDa.

Following induction at 37 °C for 6 hours, an additional culture was pelleted and either processed for isolation of inclusion bodies (see below) or  
10 processed by microfluidizing. The pellet processed for microfluidizing was resuspended in 25mM Tris-HCl/pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and collected. An aliquot was removed of the collected  
15 sample (microfluidized total lysate), and the remainder was pelleted at 20,000 x g for 20 minutes. The supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea  
20 solution (microfluidized insoluble fraction). To an aliquot of either the total soluble, or insoluble fraction was added to an equal volume of 2X Laemmeli sample buffer and β-mercaptoethanol to 5% final concentration. The samples were then analyzed by  
25 SDS-PAGE. A significant amount of recombinant huOPG met[32-401] gene product appeared to be found in the insoluble fraction.

To purify the recombinant protein, inclusion bodies were purified as follows: Bacterial cells were  
30 separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water. This  
35 suspension was transferred to a stainless steel cup

cooled in ice and subjected to sonic disruption using a Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts). The sonicated cell suspension was centrifuged in a Beckman Optima TLX 5 ultracentrifuge equipped with a TLA 100.3 rotor at 195,000  $\times$  g for 5 to 10 minutes at 23°C. The supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle. The pellets were collected by scraping with a micro spatula and 10 transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended. The volume was increased to 19.5 ml by the addition of 15 Percoll solution, mixed, and distributed into 3 Beckman Quick-Seal tubes (13  $\times$  32 mm). Tubes were sealed according to manufacturers instructions. The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600  $\times$  g), 30 minutes. The tubes were examined for 20 the appropriate banding pattern. To recover the refractile bodies, gradient fractions were recovered and pooled, then diluted with water. The inclusion bodies were pelleted by centrifugation, and the protein concentration estimated following SDS-PAGE.

25 An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemlli sample buffer with 5%  $\beta$ -mercaptoethanol and resolved on a SDS-PAGE gel and the isolated inclusion bodies provide a highly purified recombinant huOPG[32-401] gene product. 30 The major ~42 kDa band observed after resolving inclusion bodies on a SDS-polyacrylamide gel was excised from a separate gel and the N-terminal amino acid sequence determined essentially as described (Matsudaira *et al.* J. Biol. Chem. 262, 10-35 (1987)). 35 The following sequence was determined after 19 cycles:

NH<sub>2</sub> -MDEETSHQLLCDKCPPGTY-COOH (SEQ ID NO:62)

This sequence was found to be identical to the first 19 amino acids encoded by the pAMG21 Hu-OPG met[32-401] expression vector, produced by a methionine residue  
5 provided by the bacterial expression vector.

C. Human OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. Isolated plasmid DNA of pAMG21-huOPG met[32-401] (see Section B) was cleaved with KpnI and BamHI restriction endonucleases and the resulting fragments were resolved on an agarose gel. The B fragment (about 1064 bp fragment) was isolated from the gel using standard methodology. Synthetic oligonucleotides (oligos) #1267-06 and #1267-07 were phosphorylated individually and allowed to form an oligo linker duplex, which contained NdeI and KpnI cohesive ends, using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated oligo linker containing NdeI and KpnI cohesive ends and the isolated about 1064 bp fragment of pAMG21-huOPG met[32-401] digested with KpnI and BamHI restriction endonucleases were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard recombinant DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] gene.

## Oligo #1267-06:

5'-TAT GGA AAC TTT TCC TCC AAA ATA TCT TCA TTA TGA TGA AGA AAC TTC  
30 TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC GGG TAC-3'  
(SEQ ID NO:63)

## Oligo #1267-07:

5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAG AAG TTT CTT CAT  
CAT AAT GAA GAT ATT TTG GAG GAA AAG TTT CCA-3'  
35 (SEQ ID NO:64)

Cultures of pAMG21-huOPG-met[22-401] in E. coli host 393 were placed in 2XYT media containing 20 µg/ml kanamycin and were incubated at 30°C prior to induction. Induction of recombinant gene product expression from the luxPR promoter of vector pAMG21 was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and incubation at either 30°C or 37°C for a further 6 hours. After 6 hours, bacterial cultures were pelleted by centrifugation (=30°C I+6 or 37°C I+6). Bacterial cultures were also either pelleted just prior to induction (=30°C PreI) or alternatively no autoinducer was added to a separate culture which was allowed to incubate at 30°C for a further 6 hours to give an uninduced (UI) culture (=30°C UI). Bacterial pellets of either 30°C PreI, 30°C UI, 30°C I+6, or 37°C I+6 cultures were resuspended, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Section B. Polyacrylamide gels were either stained with coomassie blue and/or Western transferred to nitrocellulose and immunoprobed with rabbit anti-mu OPG-Fc polyclonal antibody as described in Example 10. The level of gene product following induction compared to either an uninduced (30°C UI) or pre-induction (30°C PreI) sample.

D. Murine OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of the murine (mu) OPG (OPG) polypeptide was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1257-16 and #1257-15 as primers, plasmid pRcCMV-Mu OPG DNA as a template and thermocycling conditions as described in Section B. The PCR product was purified and cleaved with KpnI and

BamHI restriction endonucleases as described in Section B. Synthetic oligos #1260-61 and #1260-82 were phosphorylated individually and allowed to form an oligo linker duplex with NdeI and KpnI cohesive ends 5 using methods described in Section B. The synthetic linker duplex utilized *E. coli* codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1260-61 and #1260-82 containing NdeI and KpnI cohesive ends and the KpnI and 10 BamHI digested and purified PCR product generated using oligo primers #1257-16 and #1257-15 were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into *E. coli* host 393 by electroporation 15 utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG met[22-401] gene.

Expression of recombinant muOPG met[22-401] polypeptide from cultures of 393 cells harboring plasmid pAMG21-MuOPG met[22-401] following induction was determined using methods described in Section C.

Oligo #1257-15:

20 5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTC ACG GAT TGA AC-3'  
25 (SEQ ID NO:65)

Oligo #1257-16:

5'-GTG CTC CTG GTA CCT ACC TAA AAC AGC ACT GCA CAG TG-3'  
(SEQ ID NO:66)

Oligo #1260-61:

30 5'-TAT GGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA TCC GGA AAC TGG  
TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC GGG TAC-3'  
(SEQ ID NO:67)

Oligo #1260-82:

35 5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CCG GAT CGT  
AAT GCA GGT ATT TTG GAG GCA GAG TTT CCA-3'  
(SEQ ID NO:68)

E. Murine OPG met[32-401]

A DNA sequence coding for an N-terminal methionine and amino acids 32 through 401 of murine OPG was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. To accomplish this, Synthetic oligos #1267-08 and #1267-09 were phosphorylated individually and allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized *E. coli* codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1267-08 and #1267-09 containing NdeI and KpnI cohesive ends, and the KpnI and BamHI digested and purified PCR product described earlier (see Section D), was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[32-401] gene.

Expression of recombinant muOPG-met [32-401] polypeptide from cultures of 393 cells harboring the pAMG21 recombinant plasmid following induction was determined using methods described in Section C.

Oligo #1267-08:

5'-TAT GGA CCC AGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC  
GGG TAC-3' (SEQ ID NO:69)

Oligo #1267-09:

30 5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CTG GGT  
CCA-3' (SEQ ID NO:70)

F. Murine OPG met-lys[22-401]

A DNA sequence coding for an N-terminal methionine followed by a lysine residue and amino acids 22 through 401 of murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as

follows. Synthetic oligos #1282-95 and #1282-96 were phosphorylated individually and allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1282-95 and #1282-96 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product described in Section D was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG-Met-Lys[22-401] gene.

Expression of recombinant MuOPG Met-Lys[22-401] polypeptide from transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1282-95:

5'-TAT GAA AGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA TCC GGA AAC  
TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC GGG TAC-3'  
(SEQ ID NO:71)

Oligo #1282-96:

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CCG GAT CGT  
AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT TCA-3' (SEQ ID NO:72)  
G. Murine OPG met-lys-(his)<sub>7</sub>[22-401]

A DNA sequence coding for N-terminal residues Met-Lys-His-His-His-His-His (=MKH) followed by amino acids 22 through 401 of Murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-50 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section

B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases and purified. The NdeI and BamHI digested and purified PCR 5 product generated using oligo primers #1300-50 and #1257-15 was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the 10 manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing performed to verify the DNA sequence of the muOPG-MKH[22-401] gene.

Expression of recombinant MuOPG-MKH[22-401] polypeptide from transformed 393 cultures harboring the 15 recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1300-50:  
5'-GTT CTC CTC ATA TGA AAC ATC ATC ACC ACC ATC ATG AAA CTC TGC  
CTC CAA AAT ACC TGC ATT ACG AT-3' (SEQ ID NO:73)

Oligo #1257-15: see Section D

H. Murine OPG met-lys[22-401](his)7

A DNA sequence coding for a N-terminal met-lys, amino acids 22 through 401 murine OPG, and seven histidine residues following amino acid 401 (=muOPG 25 MK[22-401]-H7), was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-49 and #1300-51 as primers and pAMG21-muOPG met[22-401] DNA as template. Thermocycling conditions 30 were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was 35 directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation was

transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG 5 MK[22-401]-H7 gene.

Expression of the recombinant muOPG MK-[22-401]-H7 polypeptide from a transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

10 Oligo #1300-49:

5'-GTT CTC CTC ATA TGA AAG AAA CTC TGC CTC CAA AAT ACC TGC A-3'  
(SEQ ID NO:74)

Oligo #1300-51:

5'-TAC GCA CTG GAT CCT TAA TGA TGG TGA TGG TGA TGT AAG CAG CTT  
15 ATT TTC ACG GAT TGA ACC TGA TTC CCT A-3' (SEQ ID NO:75)

I. Murine OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic 20 expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-74 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose 25 gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The 30 ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[27-401] gene.

35 Expression of recombinant muOPG-met[27-401] polypeptide from a transfected 393 culture harboring

the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo#1309-74:

5'-GTT CTC CTC ATA TGA AAT ACC TGC ATT ACG ATC CGG AAA CTG GTC AT-  
5 3' (SEQ ID NO:76)

Oligo#1257-15: See Section D

J. Human OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of human OPG was placed  
10 under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-75 and #1309-76 as primers and plasmid pAMG21-huOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section  
15 B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with AseI and BamHI restriction endonucleases, and purified. The AseI and BamHI digested and purified PCR product above was directionally inserted between the  
20 NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to  
25 verify the DNA sequence of the huOPG-met[27-401] gene.

Expression of the recombinant huOPG-met[27-401] polypeptide following induction of from transfected 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

30 Oligo #1309-75:

5'-GTT CTC CTA TTA ATG AAA TAT CTT CAT TAT GAT GAA GAA ACT T-3'  
(SEQ ID NO:77)

Oligo #1309-76:

5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTT ACT GAT T-3'

35 (SEQ ID NO:78)

K. Murine OPG met[22-180]

A DNA sequence coding for a N-terminal methionine and amino acids 22 through 180 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-72 and #1309-73 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[22-180] gene.

Expression of recombinant muOPG-met[22-180] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1309-72:

25 5'-GTT CTC CTC ATA TGG AAA CTC TGC CTC CAA AAT ACC TGC A-3'  
(SEQ ID NO:79)

Oligo #1309-73:

5'-TAC GCA CTG GAT CCT TAT GTT GCA TTT CCT TTC TGA ATT AGC A-3'  
(SEQ ID NO:80)

30 L. Murine OPG met[27-180]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 180 of murine OPG was placed under the control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed 35 using oligonucleotides #1309-74 (see Section I) and #1309-73 (see Section K) as primers and plasmid pAMG21-

muOPG met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG met[27-180] gene.

Expression of recombinant muOPG met[27-180] polypeptide from cultures of transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

M. Murine OPG met[22-189] and met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 189, or 22 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1337-92 and #1337-93 (=muOPG-189 linker) or #1333-57 and #1333-58 (=muOPG-194 linker) were phosphorylated individually and allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-muOPG-met[22-401] was cleaved with KpnI and BspEI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~413 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1337-92 and #1337-93 (muOPG-189 linker) or oligos #1333-57 and #1333-58 (muOPG-194 linker) containing BspEI and BamHI

cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites 5 of pAMG21-muOPG met[22-401] using standard methodology. Each ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify 10 the DNA sequence of either the muOPG-met[22-189] or muOPG-met[22-194] genes.

Expression of recombinant muOPG-met[22-189] and muOPG-met[22-194] polypeptides from recombinant pAMG21 plasmids transformed into 393 cells was determined 15 using methods described in Section C.

Oligo #1337-92:

5'-CCG GAA ACA GAT AAT GAG-3' (SEQ ID NO:81)

Oligo #1337-93:

5'-GAT CCT CAT TAT CTG TTT-3' (SEQ ID NO:82)

20 Oligo #1333-57:

5'-CCG GAA ACA GAG AAG CCA CGC AAA AGT AAG-3'  
(SEQ ID NO:83)

Oligo #1333-58:

25 5'-GAT CCT TAC TTT TGC GTG GCT TCT CTG TTT-3'  
(SEQ ID NO:84)

N. Murine OPG met[27-189] and met[27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 189, or 27 through 194 of murine OPG was placed under control of the lux 30 PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers either "muOPG-189 linker" or "muOPG-194 linker" (see Section M) containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG- 35 met[22-401] digested with KpnI and BspEI restriction endonucleases were directionally inserted between the

KpnI and BamHI sites of plasmid pAMG21-muOPG-met[27-401] using standard methodology. Each ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were 5 selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the muOPG met[27-189] or muOPG met[27-194] genes.

Expression of recombinant muOPG met[27-189] and muOPG met[27-194] following induction of 393 cells 10 harboring recombinant pAMG21 plasmids was determined using methods described in Section C.

O. Human OPG met[22-185], met[22-189], met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 185, 22 through 189, 15 or 22 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1331-87 and #1331-88 (=huOPG-185 linker), #1331-89 and #1331-90 (=huOPG- 20 189 linker), or #1331-91 & #1331-92 (=huOPG-194 linker) were phosphorylated individually and each allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-huOPG-met[27-401] was restricted with KpnI and NdeI 25 restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~407 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1331-87 and #1331-88 30 (huOPG-185 linker), oligos #1331-89 and #1331-90 (huOPG-189 linker), or oligos #1331-91 and #1331-92 (huOPG-194 linker) [each linker contains NdeI and BamHI cohesive ends], and the isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and 35 NdeI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of plasmid

pAMG21-huOPG-met[22-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the huOPG-met[22-185], huOPG-met[22-189], or huOPG-met[22-194] genes.

Expression of recombinant huOPG-met[22-185], huOPG-met[22-189] or huOPG-met[22-194] in transformed 10 393 cells harboring recombinant pAMG21 plasmids following induction was determined using methods described in Section C.

Oligo #1331-87:

5'-TAT GTT AAT GAG-3' (SEQ ID NO:85)

15 Oligo #1331-88:

5'-GAT CCT CAT TAA CA-3' (SEQ ID NO:86)

Oligo #1331-89:

5'-TAT GTT CCG GAA ACA GTT AAG-3' (SEQ ID NO:87)

Oligo #1331-90:

20 5'-GAT CCT TAA CTG TTT CCG GAA CA-3' (SEQ ID NO:88)

Oligo #1331-91:

5'-TAT GTT CCG GAA ACA GTG AAT CAA CTC AAA AAT AAG-3'

(SEQ ID NO:89)

Oligo #1331-92:

25 5'-GAT CCT TAT TTT TGA GTT GAT TCA CTG TTT CCG GAA CA-3'  
(SEQ ID NO:90)

P. Human OPG met[27-185], met[27-189], met [27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 185, 27 through 189, 30 or 27 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers "huOPG-185 linker", "huOPG-189 linker", or "huOPG-194 linker" (See Section 35 O) each containing NdeI and BamHI cohesive ends, and the isolated ~407 bp B fragment of plasmid pAMG21-

huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases (See Section O) were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[27-401] (See Section J)

5 using standard methodology. Each ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA isolated, and DNA sequencing performed to verify the DNA sequence of either the

10 huOPG-met[27-185], huOPG-met[27-189], or huOPG-met[27-194] genes.

Expression of recombinant huOPG-met[27-185], huOPG-met[27-189], and huOPG-met[27-194] from recombinant pAMG21 plasmids transformed into 393 cells

15 was determined using methods described in Section C.

O. Murine OPG met[27-401] (P33E, G36S, A45P)

A DNA sequence coding for an N-terminal methionine and amino acids 27 through 48 of human OPG followed by amino acid residues 49 through 401 of murine OPG was

20 placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows.

Purified plasmid DNA of pAMG21-huOPG-met[27-401] (See Section J) was cleaved with AatII and KpnI restriction endonucleases and a ~1075 bp B fragment isolated from

25 an agarose gel using standard recombinant DNA methodology. Additionally, plasmid pAMG21-muOPG-met[22-401] DNA (See Section D) was digested with KpnI and BamHI restriction endonucleases and the ~1064 bp B fragment isolated as described above. The isolated

30 ~1075 bp pAMG21-huOPG-met[27-401] restriction fragment containing AatII & KpnI cohesive ends (see above), the ~1064 bp pAMG21-muOPG-met[22-401] restriction fragment containing KpnI and BamHI sticky ends and a ~5043 bp restriction fragment containing AatII and BamHI

35 cohesive ends and corresponding to the nucleic acid sequence of pAMG21 between AatII & BamHI were ligated

using standard recombinant DNA methodology. The ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, and the presence of the 5 recombinant insert in the plasmid verified using standard DNA methodology. muOPG-27-401 (P33E, G36S, A45P) gene. Amino acid changes in muOPG from proline-33 to glutamic acid-33, glycine-36 to serine-36, and alanine-45 to proline-45, result from replacement of 10 muOPG residues 27 through 48 with huOPG residues 27 through 48.

Expression of recombinant muOPG-met[27-401] (P33E, G36S, A45P) from transformed 393 cells harboring the recombinant pAMG21 plasmid was determined using methods 15 described in Section C.

R. Murine OPG met-lys-(his)<sub>7</sub>-ala-ser-(asp)<sub>4</sub>-lys[22-401]  
(A45T)

A DNA sequence coding for an N-terminal His tag and enterokinase recognition sequence which is (NH<sub>2</sub> to 20 COOH terminus): Met-Lys-His-His-His-His-His-Ala-Ser-Asp-Asp-Asp-Lys (=HEK), followed by amino acids 22 through 401 of the murine OPG polypeptide was placed under control of the lac repressor regulated Ps4 promoter as follows. pAMG22-His (See Section A) was 25 digested with NheI and BamHI restriction endonucleases, and the large fragment (the A fragment) isolated from an agarose gel using standard recombinant DNA methodology. Oligonucleotides #1282-91 and #1282-92 were phosphorylated individually and allowed to form an 30 oligo linker duplex using methods previously described (See Section B). The phosphorylated linker duplex formed between oligos #1282-91 and #1282-92 containing NheI and KpnI cohesive ends, the KpnI and BamHI digested and purified PCR product described (see 35 Section D), and the A fragment of vector pAMG22-His

digested with NheI and BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into E. coli host GM120 by electroporation utilizing the manufacturer's protocol. Clones were 5 selected, plasmid DNA isolated and DNA sequencing performed to verify the DNA sequence of the muOPG-HEK[22-401] gene. DNA sequencing revealed a spurious mutation in the natural muOPG sequence that resulted in a single amino acid change of Alanine-45 of muOPG 10 polypeptide to a Threonine.

Expression of recombinant muOPG-HEK[22-401] (A45T) from GM120 cells harboring the recombinant pAMG21 plasmid was determined using methods similar to those described in Section C, except instead of addition of 15 the synthetic autoinducer, IPTG was added to 0.4 mM final to achieve induction.

Oligo #1282-91:

5'-CTA GCG ACG ACG ACG ACA AAG AAA CTC TGC CTC CAA AAT ACC TGC ATT  
ACG ATC CGG AAA CTG GTC ATC AGC TGC TGT GTG ATA AAT GTG CTC CGG  
20 GTA C-3' (SEQ ID NO:91)

Oligo #1282-92:

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CCG GAT CGT  
AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT TGT CGT CGT CGT CG-3'  
(SEQ ID NO:92)

25 S. Human OPG met-arg-gly-ser-(his)<sub>6</sub>[22-401]

Eight oligonucleotides (1338-09 to 1338-16 shown below) were designed to produce a 175 base fragment as overlapping, double stranded DNA. The oligos were annealed, ligated, and the 5' and 3' oligos were used 30 as PCR primers to produce large quantities of the 175 base fragment. The final PCR gene products were digested with restriction endonucleases ClaI and KpnI to yield a fragment which replaces the N-terminal 28 codons of human OPG. The ClaI and KpnI digested PCR 35 product was inserted into pAMG21-huOPG [27-401] which had also been cleaved with ClaI and KpnI. Ligated DNA

was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence.

5 Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Expression of huOPG Met-Arg-

10 Gly-Ser-(His)<sub>6</sub> [22-401] resulting in the formation of large inclusion bodies and the protein was localized to the insoluble (pellet) fraction.

1338-09:

ACA AAC ACA ATC GAT TTG ATA CTA GA (SEQ ID NO:93)

15 1338-10:

TTT GTT TTA ACT AAT TAA AGG AGG AAT AAA ATA TGA GAG GAT CGC ATC AC  
(SEQ ID NO:94)

1338-11:

CAT CAC CAT CAC GAA ACC TTC CCG CCG AAA TAC CTG CAC TAC GAC GAA GA  
20 (SEQ ID NO:95)

1338-12:

AAC CTC CCA CCA GCT GCT GTG CGA CAA ATG CCC GCC GGG TAC CCA AAC A  
(SEQ ID NO:96)

1338-13:

25 TGT TTG GGT ACC CGG CGG GCA TTT GT (SEQ ID NO:97)

1338-14:

CGC ACA GCA GCT GGT GGG AGG TTT CTT CGT CGT AGT GCA GGT ATT TCG GC  
(SEQ ID NO:98)

1338-15:

30 GGG AAG GTT TCG TGA TGG TGA TGG TGA TGC GAT CCT CTC ATA TTT TAT T  
(SEQ ID NO:99)

1338-16:

CCT CCT TTA ATT AGT TAA AAC AAA TCT AGT ATC AAA TCG ATT GTG TTT GT  
(SEQ ID NO:100)

35 T. Human OPG met-lys[22-401] and met(lys)<sub>3</sub>[22-401]

To construct the met-lys and met-(lys)<sub>3</sub> versions of human OPG[22-401], overlapping oligonucleotides were designed to add the appropriate number of lysine

residues. The two oligos for each construct were designed to overlap, allowing two rounds of PCR to produce the final product. The template for the first PCR reaction was a plasmid DNA preparation containing 5 the human OPG 22-401 gene. The first PCR added the lysine residue(s). The second PCR used the product of the first round and added sequence back to the first restriction site, ClaI.

The final PCR gene products were digested with 10 restriction endonucleases ClaI and KpnI, which replace the N-terminal 28 codons of hu OPG, and then ligated into plasmid pAMG21-hu OPG [27-401] which had been also digested with the two restriction endonucleases.

Ligated DNA was transformed into competent host cells 15 of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell 20 lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Neither construct had a detectable level of protein expression and inclusion bodies were not visible. The DNA 25 sequences were confirmed by DNA sequencing.

Oligonucleotide primers to prepare Met-Lys huOPG[22-401]:

1338-17:

ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT AAA GGA  
30 GGA ATA AAA TG (SEQ ID NO:101)

1338-18:

CTA ATT AAA GGA GGA ATA AAA TGA AAG AAA CTT TTC CTC CAA AAT ATC  
(SEQ ID NO:102)

1338-20:

35 TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID NO:103)

Oligonucleotide primers to prepare Met-(Lys)<sub>3</sub>-huOPG[22-401]:

1338-17:

5 ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT AAA GGA  
GGA ATA AAA TG (SEQ ID NO:104)

1338-19:

CTA ATT AAA GGA GGA ATA AAA TGA AAA AAA AAG AAA CTT TTC CTC CAA  
AAT ATC (SEQ ID NO:105)

1338-20:

10 TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID NO:106)

U. Human and Murine OPG [22-401]/Fc Fusions

Four OPG-Fc fusions were constructed where the Fc region of human IgG1 was fused at the N-terminus of either human or murine Osteoprotegerin amino acids 22 to 401 (referred to as Fc/OPG [22-401]) or at the C-terminus (referred to as OPG[22-401]/Fc). Fc fusions were constructed using the fusion vector pFc-A3 described in Example 7.

All fusion genes were constructed using standard PCR technology. Template for PCR reactions were plasmid preparations containing the target genes. Overlapping oligos were designed to combine the C-terminal portion of one gene with the N terminal portion of the other gene. This process allows fusing the two genes together in the correct reading frame after the appropriate PCR reactions have been performed. Initially one "fusion" oligo for each gene was put into a PCR reaction with a universal primer for the vector carrying the target gene. The complimentary "fusion" oligo was used with a universal primer to PCR the other gene. At the end of this first PCR reaction, two separate products were obtained, with each individual gene having the fusion site present, creating enough overlap to drive the second round of PCR and create the desired fusion. In the second round of PCR, the first two PCR products were combined along with universal primers and via the

overlapping regions, the full length fusion DNA sequence was produced.

The final PCR gene products were digested with restriction endonucleases XbaI and BamHI, and then 5 ligated into the vector pAMG21 having been also digested with the two restriction endonucleases. Ligated DNA was transformed into competent host cells of *E. coli* strain 393. Clones were screened for the ability to produce the recombinant protein product and 10 to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate, sonic pellet, and supernatant were analyzed for expression of the fusion by Coomassie stained PAGE gels 15 and Western analysis with murine anti-OPG antibody.

Fc/huOPG [22-401]

Expression of the Fc/hu OPG [22-401] fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The cells have very large 20 inclusion bodies, and the majority of the product is in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

1318-48:

CAG CCC GGG TAA AAT GGA AAC GTT TCC TCC AAA ATA TCT TCA TT  
25 (SEQ ID NO:107)

1318-49:

CGT TTC CAT TTT ACC CGG GCT GAG CGA GAG GCT CTT CTG CGT GT  
(SEQ ID NO:108)

Fc/muOPG [22-401]

30 Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The cells have very large inclusion bodies, and the majority of the product is in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc 35 fusion:

1318-50:

CGC TCA GCC CGG GTA AAA TGG AAA CGT TGC CTC CAA AAT ACC TGC  
(SEQ ID NO:109)

1318-51:

CCA TTT TAC CCG GGC TGA GCG AGA GGC TCT TCT GCG TGT  
5 (SEQ ID NO:110)

muOPG [22-401]/Fc :

Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The amount of recombinant product was less than the OPG fusion 10 proteins having the Fc region in the N terminal position. Obvious inclusion bodies were not detected. Most of the product appeared to be in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

15 1318-54:

GAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC (SEQ ID NO:111)

1318-55:

CAG CTG CAG CTA AGC AGC TTA TTT TCA CGG ATT G (SEQ ID NO:112)

huOPG [22-401]/Fc

20 Expression of the fusion peptide was not detected on a Coomassie stained gel, although a faint Western positive signal was present. Obvious inclusion bodies were not detected. The following primers were used to prepare this OPG-Fc fusion:

25 1318-52:

AAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC (SEQ ID NO:113)

1318-53:

CAG CTG CAG CTA AGC AGC TTA TTT TTA CTG ATT GG (SEQ ID NO:114)

V. Human OPG met[22-401]-Fc fusion (P25A)

30 This construct combines a proline to alanine amino acid change at position 25 (P25A) with the huOPG met[22-401]-Fc fusion. The plasmid was digested with restriction endonucleases ClaI and KpnI, which removes the N-terminal 28 codons of the gene, and the resulting 35 small (less than 200 base pair) fragment was gel purified. This fragment containing the proline to

alanine change was then ligated into plasmid pAMG21-huOPG [22-401]-Fc fusion which had been digested with the two restriction endonucleases. The ligated DNA was transformed into competent host cells of E. coli strain 5 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were 10 analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. The expression level of the fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The protein was in the insoluble 15 (pellet) fraction. The cells had large inclusion bodies.

W. Human OPG met[22-401] (P25A)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the 20 proline at position 25 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-84 and 1289-85 were annealed to form an oligo linker duplex with XbaI and KpnI cohesive 25 ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker also included an SpeI restriction site which was not present in the original sequence. The linker duplex was directionally inserted between the XbaI and KpnI 30 sites in pAMG21-huOPG-22-401 using standard methods. The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing 35 was performed to verify the DNA sequence of the HuOPG-

Met[22-401] (P25A) gene. The following oligonucleotides were used to generate the XbaI - KpnI linker:

Oligo #1289-84:

5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC AAA ATA TCT TCA  
5 TTA TGA TGA AGA AAC TAG TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC  
GGG TAC -3' (SEQ ID NO:115)

Oligo #1289-85:

5' - CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAC TAG TTT CTT CAT CAT  
AAT GAA GAT ATT TTG GAG CAA AAG TTT CCA TAT GTT ATT CCT CCT T-3'  
10 (SEQ ID NO:116)

X. Human OPG met[22-401] (P26A) and (P26D)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 26 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-86 and 1289-87 were annealed to form an oligo linker duplex with XbaI and SpeI cohesive ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker duplex was directionally inserted between the XbaI and SpeI sites in pAMG21-huOPG[22-401] (P25A) using standard methods. The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] (P26A) gene. One of the clones sequenced was found to have the proline at position 26 substituted by aspartic acid rather than alanine, and this clone was designated huOPG-met[22-401] (P26D). The following oligonucleotides were used to generate the XbaI - SpeI linker:

Oligo #1289-86:

35 5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC TAA ATA TCT  
TCA TTA TGA TGA AGA AA - 3' (SEQ ID NO:117)

Oligo #1289-87:

5' - CTA GTT TCT TCA TCA TAA TGA AGA TAT TTA GCA GGA AAA GTT TCC  
ATA TGT TAT TCC TCC TT - 3' (SEQ ID NO:118)

Y. Human OPG met[22-194] (P25A)

5 A DNA sequence coding for an N-terminal methionine and amino acids 22 through 194 of human OPG with the proline at position 25 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows:

10 The plasmids pAMG21-huOPG[27-194] and pAMG21-huOPG[22-401] (P25A) were each digested with KpnI and BamHI endonucleases. The 450 bp fragment was isolated from pAMG21-huOPG[27-194] and the 6.1 kbp fragment was isolated from pAMG21-huOPG[22-401] (P25A). These

15 fragments were ligated together and introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA

20 sequence of the huOPG-Met[22-194] (P25A) gene.

**EXAMPLE 9**

Association of OPG Monomers

CHO cells engineered to overexpress muOPG [22-401] were used to generate conditioned media for the analysis of secreted recombinant OPG using rabbit polyclonal anti-OPG antibodies. An aliquot of conditioned media was concentrated 20-fold, then analysed by reducing and non-reducing SDS-PAGE (Figure 15). Under reducing conditions, the protein migrated as a Mr 50-55 kd polypeptide, as would be predicted if the mature product was glycosylated at one or more of its consensus N-linked glycosylation sites. Surprisingly, when the same samples were analysed by non-reducing SDS-PAGE, the majority of the protein migrated as an approximately 100 kd polypeptide, twice the size of the reduced protein. In addition, there was

a smaller amount of the Mr 50-55 kd polypeptide. This pattern of migration on SDS-PAGE was consistent with the notion that the OPG product was forming dimers through oxidation of a free sulfhydryl group(s).

5       The predicted mature OPG polypeptide contains 23 cysteine residues, 18 of which are predicted to be involved in forming intrachain disulfide bridges which comprise the four cysteine-rich domains (Figure 12A). The five remaining C-terminal cysteine residues are not  
10 involved in secondary structure which can be predicted based upon homology with other TNFR family members. Overall there is a net uneven number of cysteine residues, and it is formally possible that at least one residue is free to form an intermolecular disulfide  
15 bond between two OPG monomers.

To help elucidate patterns of OPG kinesis and monomer association, a pulse-chase labelling study was performed. CHO cells expressing muOPG [22-401] were metabolically labelled as described above in serum-free  
20 medium containing  $^{35}\text{S}$  methionine and cysteine for 30 min. After this period, the media was removed, and replaced with complete medium containing unlabelled methionine and cysteine at levels approximately 2,000-fold excess to the original concentration of  
25 radioactive amino acids. At 30 min, 1hr, 2 hr, 4 hr, 6 hr and 12 hr post addition, cultures were harvested by the removal of the conditioned media, and lysates of the conditioned media and adherent monolayers were prepared. The culture media and cell lysates were  
30 clarified as described above, and then immunoprecipitated using anti-OPG antibodies as described above. After the immunoprecipitates were washed, they were released by boiling in non-reducing SDS-PAGE buffer then split into two equal halves. To  
35 one half, the reducing agent  $\beta$ -mercaptoethanol was added

to 5% (v/v) final concentration, while the other half was maintained in non-reducing conditions. Both sets of immunoprecipitates were analysed by SDS-PAGE as described above, then processed for autoradiography and exposed to film. The results are shown in Figure 16.

The samples analysed by reducing SDS-PAGE are depicted in the bottom two panels. After synthesis, the OPG polypeptide is rapidly processed to a slightly larger polypeptide, which probably represents modification by N-linked glycosylation. After approximately 1-2 hours, the level of OPG in the cell decreases dramatically, and concomitantly appears in the culture supernatant. This appears to be the result of the vectoral transport of OPG from the cell into the media over time, consistent with the notion that OPG is a naturally secreted protein. Analysis of the same immunoprecipitates under nonreducing conditions reveals the relationship between the formation of OPG dimers and secretion into the conditioned media (Figure 16, upper panels). In the first 30-60 minutes, OPG monomers are processed in the cell by apparent glycosylation, followed by dimer formation. Over time, the bulk of OPG monomers are driven into dimers, which subsequently disappear from the cell. Beginning about 60 minutes after synthesis, OPG dimers appear in the conditioned media, and accumulate over the duration of the experiment. Following this period, OPG dimers are formed, which are then secreted into the culture media. OPG monomers persist at a low level inside the cell over time, and small amounts also appear in the media. This does not appear to be the result of breakdown of covalent OPG dimers, but rather the production of sub-stoichiometric amounts of monomers in the cell and subsequent secretion.

Recombinantly produced OPG from transfected CHO cells appears to be predominantly a dimer. To determine

if dimerization is a natural process in OPG synthesis, we analysed the conditioned media of a cell line found to naturally express OPG. The CTLL-2 cell line, a murine cytotoxic T lymphocytic cell line (ATCC accession no. TIB-214), was found to express OPG mRNA in a screen of tissue and cell line RNA. The OPG transcript was found to be the same as the cloned and sequenced 2.5-3.0 kb RNA identified from kidney and found to encode a secreted molecule. Western blot analysis of conditioned media obtained from CTLL-2 cells shows that most, if not all, of the OPG secreted is a dimer (Figure 17). This suggests that OPG dimerization and secretion is not an artifact of overexpression in a cell line, but is likely to be the main form of the product as it is produced by expressing cells.

Normal and transgenic mouse tissues and serum were analysed to determine the nature of the OPG molecule expressed in OPG transgenic mice. Since the rat OPG cDNA was expressed under the control of a hepatocyte control element, extracts made from the parenchyma of control and transgenic mice under non-reducing conditions were analysed (Figure 18). In extract from transgenic, but not control mice, OPG dimers are readily detected, along with substoichiometric amounts of monomers. The OPG dimers and monomers appear identical to the recombinant murine protein expressed in the genetically engineered CHO cells. This strongly suggests that OPG dimers are indeed a natural form of the gene product, and are likely to be key active components. Serum samples obtained from control and transgenic mice were similarly analysed by western blot analysis. In control mice, the majority of OPG migrates as a dimer, while small amounts of monomer are also detected. In addition, significant amounts of a larger OPG related protein is detected, which migrates with a

relative molecular mass consistent with the predicted size of a covalently-linked trimer. Thus, recombinant OPG is expressed predominantly as a dimeric protein in OPG transgenic mice, and the dimer form may be the 5 basis for the osteopetrosis phenotype in OPG mice. OPG recombinant protein may also exist in higher molecular weight "trimeric" forms.

To determine if the five C-terminal cysteine residues of OPG play a role in homodimerization, the 10 murine OPG codons for cysteine residues 195 (C195), C202, C277, C319, and C400 were changed to serine using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as described above. The muOPG gene was subcloned between the Not I and Xba I 15 sites of the pcDNA 3.1 (+) vector (Invitrogen, San Diego, CA). The resulting plasmid, pcDNA3.1-muOPG, and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the 20 reaction is then transfected into competent *E. coli* XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to change the codon for cysteine residue 195 to serine of the murine 25 OPG gene, resulting in the production of a muOPG [22-401] C195S protein:

. 1389-19:

5' -CAC GCA AAA GTC GGG AAT AGA TGT CAC-3' (SEQ ID NO:150)

1406-38:

30 5' -GTG ACA TCT ATT CCC GAC TTT TGC GTG-3' (SEQ ID NO:151)

The following primer pairs were used to change the codon for cysteine residue 202 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C202S protein:

35 1389-21:

5' -CAC CCT GTC GGA AGA GGC CTT CTT C-3' (SEQ ID NO:152)

1389-22:

5' -GAA GAA GGC CTC TTC CGA CAG GGT G-3' (SEQ ID NO:153)

The following primer pairs were used to change the codon for cysteine residue 277 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C277S protein:

1389-23:

5' -TGA CCT CTC GGA AAG CAG CGT GCA-3' (SEQ ID NO:154)

1389-24:

10 5' -TGC ACG CTG CTT TCC GAG AGG TCA-3' (SEQ ID NO:155)

The following primer pairs were used to change the codon for cysteine residue 319 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C319S protein:

15 1389-17:

5' -CCT CGA AAT CGA GCG AGC AGC TCC-3' (SEQ ID NO:156)

1389-18:

5' -CGA TTT CGA GGT CTT TCT CGT TCT C-3' (SEQ ID NO:157)

20 The following primer pairs were used to change the codon for cysteine residue 400 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C400S protein:

1406-72:

5' -CCG TGA AAA TAA GCT CGT TAT AAC TAG GAA TGG-3'

25 (SEQ ID NO:158)

1406-75:

5' -CCA TTC CTA GTT ATA ACG AGC TTA TTT TCA CGG-3'

(SEQ ID NO:159)

30 Each resulting muOPG [22-401] plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11.

35 Conditioned media from each transfectant was analysed

by non-reducing SDS-PAGE and western blotting with anti-OPG antibodies.

Mutation of any of the five C-terminal cysteine residues results in the production of predominantly (>90%) monomeric 55 kd OPG molecules. This strongly suggests that the C-terminal cysteine residues together play a role in OPG homodimerization.

C-terminal OPG deletion mutants were constructed to map the region(s) of the OPG C-terminal domain which are important for OPG homodimerization. These OPG mutants were constructed by PCR amplification using primers which introduce premature stop translation signals in the C-terminal region of murine OPG. The 5' oligo was designed to the MuOPG start codon (containing a HindIII restriction site) and the 3' oligonucleotides (containing a stop codon and XhoI site) were designed to truncate the C-terminal region of muOPG ending at either threonine residue 200 (CT 200), proline 212 (CT212), glutamic acid 293 (CT-293), or serine 355 (CT-355).

The following primers were used to construct muOPG [22-200]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
25 (SEQ ID NO:160)

1391-91:

5' -CCT CTC TCG AGT CAG GTG ACA TCT ATT CCA CAC TTT TGC GTG GC-3'  
(SEQ ID NO:161)

The following primers were used to construct muOPG [22-212]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
(SEQ ID NO:162)

1391-90:

35 5' -CCT CTC TCG AGT CAA GGA ACA GCA AAC CTG AAG AAG GC -3'  
(SEQ ID NO:163)

The following primers were used to construct muOPG [22-293]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
5 (SEQ ID NO:164)

1391-89:

5'- CCT CTC TCG AGT CAC TCT GTG GTG AGG TTC GAG TGG CC-3'  
(SEQ ID NO:165)

The following primers were used to construct muOPG  
10 [22-355]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
(SEQ ID NO:166)

1391-88:

15 5' CCT CTC TCG AGT CAG GAT GTT TTC AAG TGC TTG AGG GC-3'  
(SEQ ID NO:167)

Each resulting muOPG-CT plasmid containing the appropriate truncation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified  
20 from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11. The conditioned medias were also analysed by non-reducing SDS-PAGE and western blotting using anti-OPG  
25 antibodies.

Truncation of the C-terminal region of OPG effects the ability of OPG to form homodimers. CT 355 is predominantly monomeric, although some dimer is formed. CT 293 forms what appears to be equal molar amounts of monomer and dimer, and also high molecular weight aggregates. However, CT 212 and CT 200 are monomeric.

#### EXAMPLE 10

##### Purification of OPG

###### A. Purification of mammalian OPG-Fc Fusion Proteins

35 5 L of conditioned media from 293 cells expressing an OPG-Fc fusion protein were prepared as follows. A

frozen sample of cells was thawed into 10 ml of 293S media (DMEM-high glucose, 1x L-glutamine, 10% heat inactivated fetal bovine serum (FBS) and 100 ug/ml hygromycin) and fed with fresh media after one day.

5 After three days, cells were split into two T175 flasks at 1:10 and 1:20 dilutions. Two additional 1:10 splits were done to scale up to 200 T175 flasks. Cells were at 5 days post-thawing at this point. Cells were grown to near confluence (about three days) at which time serum-containing media was aspirated, cells were washed one time with 25 ml PBS per flask and 25 ml of SF media (DMEM-high glucose, 1x L-glutamine) was added to each flask. Cells were maintained at 5% CO<sub>2</sub> for three days at which point the media was harvested, centrifuged,

10 and filtered through 0.45m cellulose nitrate filters (Corning).

15

OPG-Fc fusion proteins were purified using a Protein G Sepharose column (Pharmacia) equilibrated in PBS. The column size varied depending on volume of starting media. Conditioned media prepared as described above was loaded onto the column, the column washed with PBS, and pure protein eluted using 100mM glycine pH 2.7. Fractions were collected into tubes containing 1M Tris pH 9.2 in order to neutralize as quickly as possible. Protein containing fractions were pooled, concentrated in either an Amicon Centricon 10 or Centriprep 10 and diafiltered into PBS. The pure protein is stored at -80°C.

Murine [22-401]-Fc, Murine [22-180]-Fc, Murine [22-194]-Fc, human [22-401]-Fc and human [22-201]Fc were purified by this procedure. Murine [22-185]-Fc is purified by this procedure.

B. Preparation of anti-OPG antibodies

Three New Zealand White rabbits (5-8 lbs initial wt) were injected subcutaneously with muOPG[22-401]-Fc fusion protein. Each rabbit was immunized on day 1 with

50 µg of antigen emulsified in an equal volume of Freunds complete adjuvant. Further boosts (Days 14 and 28) were performed by the same procedure with the substitution of Freunds incomplete adjuvant. Antibody titers were monitored by EIA. After the second boost, the antisera revealed high antibody titers and 25ml production bleeds were obtained from each animal. The sera was first passed over an affinity column to which murine OPG-Fc had be immobilized. The anti-OPG antibodies were eluted with Pierce Gentle Elution Buffer containing 1% glacial acetic acid. The eluted protein was then dialyzed into PBS and passed over a Fc column to remove any antibodies specific for the Fc portion of the OPG fusion protein. The run through fractions containing anti-OPG specific antibodies were dialyzed into PBS.

C. Purification of murine OPG[22-401]

Antibody Affinity Chromatography

Affinity purified anti-OPG antibodies were diafiltered into coupling buffer (0.1M sodium carbonate pH 8.3, 0.5M NaCl), and mixed with CNBr-activated sepharose beads (Pharmacia) for two hours at room temperature. The resin was then washed with coupling buffer extensively before blocking unoccupied sited with 1M ethanolamine (pH 8.0) for two hours at room temperature. The resin was then washed with low pH (0.1M sodium acetate pH 4.0, 0.5M NaCl) followed by a high pH wash (0.1M Tris-HCl pH 8.0, 0.5M NaCl). The last washes were repeated three times. The resin was finally equilibrated with PBS before packing into a column. Once packed, the resin was washed with PBS. A blank elution was performed with 0.1M glycine-HCl, pH 2.5), followed by re-equilibration with PBS.

Concentrated conditioned media from CHO cells expressing muOPG[22-410] was applied to the column at a

low flow rate. The column was washed with PBS until UV absorbance measured at 280nm returned to baseline. The protein was eluted from the column first with 0.1M glycine-HCl (pH 2.5), re-equilibrated with PBS, and 5 eluted with a second buffer (0.1M CAPS, pH 10.5), 1M NaCl). The two elution pools were diafiltered separately into PBS and sterile filtered before freezing at -20°C.

Conventional Chromatography

10 CHO cell conditioned media was concentrated 23x in an Amicon spiral wound cartridge (S10Y10) and diafiltered into 20mM tris pH 8.0. The diafiltered media was then applied to a Q-sepharose HP (Pharmacia) column which had been equilibrated with 20mM tris pH 15 8.0. The column was then washed until absorbence at 280 nm reached baseline. Protein was eluted with a 20 column volume gradient of 0-300 mM NaCl in tris pH 8.0. OPG was detected using a western blot of column fractions.

20 Fractions containing OPG were pooled and brought to a final concentration of 300 mM NaCl, 0.2 mM DTT. A NiNTA superose (Qiagen) column was equilibrated with 20mM tris pH 8.0, 300 mM NaCl, 0.2 mM DTT after which the pooled fractions were applied. The column was 25 washed with equilibration buffer until baseline absorbence was reached. Proteins were eluted from the column with a 0-30mM Imidazole gradient in equilibration buffer. Remaining proteins were washed off the column with 1M Imidazole. Again a western blot 30 was used to detect OPG containing fractions.

Pooled fractions from the NiNTA column were dialyzed into 10 mM potassium phosphate pH 7.0, 0.2mM DTT. The dialyzed pool was then applied to a ceramic hydroxyapatite column (Bio-Rad) which had been 35 equilibrated in 10mM phosphate buffer. After column washing, the protein was eluted with a 10-100 mM

potassium phosphate gradient over 20 column volumes. This was then followed by a 20 column volume gradient of 100-400 mM phosphate.

OPG was detected by coomassie blue staining of  
5 SDS-polyacrylamide gels and by western blotting. Fractions were pooled and diafiltered onto PBS and frozen at -80°C. The purified protein runs as a monomer and will remain so after diafiltration into PBS. The monomer is stable when stored frozen or at pH 5 at 4°C.  
10 However if stored at 4°C in PBS, dimers and what appears to be trimers and tetramers will form after one week.

D. Purification of human OPG met[22-401] from E. coli

The bacterial cell paste was suspended into 10 mM  
15 EDTA to a concentration of 15% (w/v) using a low shear homogenizer at 5°C. The cells were then disrupted by two homogenizations at 15,000 psi each at 5°C. The resulting homogenate was centrifuged at 5,000 x g for one hour at 5°C. The centrifugal pellet was washed by  
20 low shear homogenization into water at the original homogenization volume followed by centrifugation as before. The washed pellet was then solubilized to 15% (w/v) by a solution of (final concentration) 6 M guanidine HCl, 10 mM dithiothreitol, 10 mM TrisHCl, pH  
25 8.5 at ambient temperature for 30 minutes. This solution was diluted 30-fold into 2M urea containing 50 mM CAPS, pH 10.5, 1 mM reduced glutathione and then stirred for 72 hours at 5°C. The OPG was purified from this solution at 25°C by first adjustment to pH 4.5  
30 with acetic acid and then chromatography over a column of SP-HP Sepharose resin equilibrated with 25 mM sodium acetate, pH 4.5. The column elution was carried out with a linear sodium chloride gradient from 50 mM to 550 mM in the same buffer using 20 column volumes at a  
35 flow rate of 0.1 column volumes/minute. The peak fractions containing only the desired OPG form were

pooled and stored at 5°C or buffer exchanged into phosphate buffered saline, concentrated by ultrafiltration, and then stored at 5°C. This material was analyzed by reverse phase HPLC, SDS-PAGE, limulus 5 amebocyte lysate assay for the presence of endotoxin, and N-terminal sequencing. In addition, techniques such as mass spectrometry, pH/temperature stability, fluorescence, circular dichroism, differential scanning calorimetry, and protease profiling assays may also be 10 used to examine the folded nature of the protein.

**EXAMPLE 11**

**Biological Activity of Recombinant OPG**

Based on histology and histomorphometry, it appeared that hepatic overexpression of OPG in 15 transgenic mice markedly decreased the numbers of osteoclasts leading to a marked increase in bone tissue (see Example 4). To gain further insight into potential mechanism(s) underlying this *in vivo* effect, various forms of recombinant OPG have been tested in an *in* 20 *vitro* culture model of osteoclast formation (osteoclast forming assay). This culture system was originally devised by Udagawa (Udagawa *et al.* Endocrinology 125, 1805-1813 (1989), Proc. Natl. Acad. Sci. USA 87, 7260- 25 7264 (1990)) and employs a combination of bone marrow cells and cells from bone marrow stromal cell lines. A description of the modification of this culture system used for these studies has been previously published (Lacey *et al.* Endocrinology 136, 2367-2376 (1995)). In this method, bone marrow cells, flushed from the femurs 30 and tibiae of mice, are cultured overnight in culture media (alpha MEM with 10% heat inactivated fetal bovine serum) supplemented with 500 U/ml CSF-1 (colony stimulating factor 1, also called M-CSF), a hematopoietic growth factor specific for cells of the 35 monocyte/macrophage family lineage. Following this incubation, the non-adherent cells are collected,

subjected to gradient purification, and then cocultured with cells from the bone marrow cell line ST2 ( $1 \times 10^6$  non-adherent cells :  $1 \times 10^5$  ST2 cells/ ml media). The media is supplemented with dexamethasone (100 nM) and 5 the biologically-active metabolite of vitamin D<sub>3</sub> known as 1,25 dihydroxyvitamin D<sub>3</sub> (1,25 (OH)<sub>2</sub> D<sub>3</sub>, 10 nM). To enhance osteoclast appearance, prostaglandin E2 (250 nM) is added to some cultures. The coculture period usually ranges from 8 - 10 days and the media, with all 10 of the supplements freshly added, is renewed every 3-4 days. At various intervals, the cultures are assessed for the presence of tartrate acid phosphatase (TRAP) using either a histochemical stain (Sigma Kit # 387A, Sigma, St. Louis, MO) or TRAP solution assay. The TRAP 15 histochemical method allows for the identification of osteoclasts phenotypically which are multinucleated (> 3 nuclei) cells that are also TRAP+. The solution assay involves lysing the osteoclast-containing cultures in a citrate buffer (100 mM, pH 5.0) containing 0.1% Triton 20 X-100. Tartrate resistant acid phosphatase activity is then measured based on the conversion of p-nitrophenylphosphate (20 nM) to p-nitrophenol in the presence of 80 mM sodium tartrate which occurs during a 3-5 minute incubation at RT. The reaction is terminated 25 by the addition of NaOH to a final concentration of 0.5 M. The optical density at 405 nm is measured and the results are plotted.

Previous studies (Udagawa *et al. ibid*) using the osteoclast forming assay have demonstrated that these 30 cells express receptors for <sup>125</sup>I-calcitonin (autoradiography) and can make pits on bone surfaces, which when combined with TRAP positivity confirm that the multinucleated cells have an osteoclast phenotype. Additional evidence in support of the osteoclast 35 phenotype of the multinucleated cells that arise in

vitro in the osteoclast forming assay are that the cells express  $\alpha v$  and  $\beta 3$  integrins by immunocytochemistry and calcitonin receptor and TRAP mRNA by *in situ* hybridization (ISH).

- 5       The huOPG [22-401]-Fc fusion was purified from CHO cell conditioned media and subsequently utilized in the osteoclast forming assay. At 100 ng/ml of huOPG [22-401]-Fc, osteoclast formation was virtually 100% inhibited (Figure 19A). The levels of TRAP measured in  
10 lysed cultures in microtitre plate wells were also inhibited in the presence of OPG with an ID<sub>50</sub> of approximately 3 ng/ml (Figure 20). The level of TRAP activity in lysates appeared to correlate with the relative number of osteoclasts seen by TRAP  
15 cytochemistry (compare Figures 19A-19G and 20). Purified human IgG1 and TNF- $\alpha$  inhibitor were also tested in this model and were found to have no inhibitory or stimulatory effects suggesting that the inhibitory effects of the huOPG [22-401]-Fc were due to  
20 the OPG portion of the fusion protein. Additional forms of the human and murine molecules have been tested and the cumulative data are summarized in Table 3.

Table 3  
Effects of various OPG forms on in vitro  
osteoclast formation

	<u>OPG Construct</u>	<u>Relative Bioactivity in vitro</u>
	muOPG [22-401]-Fc	+++
	muOPG [22-194]-Fc	+++
	muOPG [22-185]-Fc	++
10	muOPG [22-180]-Fc	-
	muOPG [22-401]	+++
	muOPG [22-401] C195	+++
	muOPG [22-401] C202	+
	muOPG [22-401] C277	-
15	muOPG [22-401] C319	+
	muOPG [22-401] C400	+
	muOPG [22-185]	-
	muOPG [22-194]	++
	muOPG [22-200]	++
20	muOPG [22-212]	-
	muOPG [22-293]	+++
	muOPG [22-355]	+++
	huOPG [22-401]-Fc	+++
25	huOPG [22-201]-Fc	+++
	huOPG [22-401]-Fc P26A	+++
	huOPG [22-401]-Fc Y28F	+++
	huOPG [22-401]	+++
	huOPG [27-401]-Fc	++
30	huOPG [29-401]-Fc	++
	huOPG [32-401]-Fc	+/-
	+++ , ED <sub>50</sub> = 0.4-2 ng/ml	
	++ , ED <sub>50</sub> = 2-10 ng/ml	
35	+ , ED <sub>50</sub> = 10-100 ng/ml	
	- , ED <sub>50</sub> > 100 ng/ml	

The cumulative data suggest that murine and human OPG amino acid sequences 22-401 are fully active in vitro, when either fused to the Fc domain, or unfused. They inhibit in a dose-dependent manner and possess 5 half-maximal activities in the 2-10 ng/ml range. Truncation of the murine C-terminus at threonine residue 180 inactivates the molecule, whereas truncations at cysteine 185 and beyond have full activity. The cysteine residue located at position 185 10 is predicted to form an SS3 bond in the domain 4 region of OPG. Removal of this residue in other TNFR-related proteins has previously been shown to abrogate biological activity (Yan *et al.* (1994), *J. Biol. Chem.* 266: 12099-104). Our finding that muOPG[22-180]-Fc is 15 inactive while muOPG[22-185]-Fc is active is consistent with these findings. This suggests that amino acid residues 22-185 define a region for OPG activity.

These findings indicate that like transgenically-expressed OPG, recombinant OPG also 20 suppressed osteoclast formation as tested in the osteoclast forming assay. Time course experiments examining the appearance of TRAP+ cells,  $\beta$ 3+ cells, F480+ cells in cultures continuously exposed to OPG demonstrate that OPG blocks the appearance TRAP+ and 25  $\beta$ 3+ cells, but not F480+ cells. In contrast, TRAP+ and  $\beta$ 3+ cells begin to appear as early as day 4 following culture establishment in control cultures. Only F480+ cells can be found in OPG-treated cultures and they 30 appear to be present at qualitatively the same numbers as the control cultures. Thus, the mechanism of OPG effects in vitro appears to involve a blockade in osteoclast differentiation at a step beyond the appearance of monocyte-macrophages but before the 35 appearance of cells expressing either TRAP or  $\beta$ 3 integrins. Collectively these findings indicate that

OPG does not interfere with the general growth and differentiation of monocyte-macrophage precursors from bone marrow, but rather suggests that OPG specifically blocks the selective differentiation of osteoclasts  
5 from monocyte-macrophage precursors.

To determine more specifically when in the osteoclast differentiation pathway that OPG was inhibitory, a variation of the in vitro culture method was employed. This variation, described in (Lacey *et al. supra*), employs bone marrow macrophages as  
10 osteoclast precursors. The osteoclast precursors are derived by taking the nonadherent bone marrow cells after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000  
15 - 2,000 U/ml CSF-1. Following 4 days of culture, termed the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/ml CSF-1.  
20 This 2 day period is called the intermediate differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells ( $1 \times 10^5$  cell/ml), dexamethasone (100 nM) and 1,25 (OH)<sub>2</sub> D<sub>3</sub> (10 nM) are added for the last 8 days for what is termed the  
25 terminal differentiation period. Test agents can be added during this terminal period as well. Acquisition of phenotypic markers of osteoclast differentiation are acquired during this terminal period (Lacey *et al. ibid*).

30 huOPG [22-401]-Fc (100 ng/ml) was tested for its effects on osteoclast formation in this model by adding it during either the intermediate, terminal or, alternatively, both differentiation periods. Both TRAP cytochemistry and solution assays were performed. The  
35 results of the solution assay are shown in Figure 21.

HuOPG [22-401]-Fc inhibited the appearance of TRAP activity when added to both the intermediate and terminal or only the terminal differentiation phases. When added to the intermediate phase and then removed from the cultures by rinsing, huOPG [22-401]-Fc did not block the appearance of TRAP activity in culture lysates. The cytochemistry results parallel the solution assay data. Collectively, these observations indicate that huOPG [22-401]-Fc only needs to be present during the terminal differentiation period for it to exert its all of its suppressive effects on osteoclast formation.

B. In vivo IL-1- $\alpha$  and IL-1- $\beta$  challenge experiments

IL-1 increases bone resorption both systemically and locally when injected subcutaneously over the calvaria of mice (Boyce *et al.* (1989), *Endocrinology* 125: 1142-50). The systemic effects can be assessed by the degree of hypercalcemia and the local effects histologically by assessing the relative magnitude of the osteoclast-mediated response. The aim of these experiments was to determine if recombinant muOPG [22-401]-Fc could modify the local and/or systemic actions of IL-1 when injected subcutaneously over the same region of the calvaria as IL-1.

IL-1  $\beta$  experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group: IL-1 treated animals (mice received 1 injection/day of 2.5 ug of IL-1- $\beta$ ); Low dose muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 1  $\mu$ g of muOPG [22-401]-Fc); Low dose muOPG [22-401]-Fc and IL-1- $\beta$ ; High dose muOPG [22-401]-Fc treated animals (mice receive 3 injections/day of 10  $\mu$ g muOPG [22-401]-Fc); High dose muOPG [22-401]-Fc and

IL-1- $\beta$ . All mice received the same total number of injections of either active factor or vehicle (0.1% bovine serum albumin in phosphate buffered saline). All groups are sacrificed on the day after the last injection. The weights and blood ionized calcium levels are measured before the first injections, four hours after the second injection and 24 hours after the third IL-1 injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin sectioning.

IL-1  $\alpha$  experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group; IL-1- $\alpha$  treated animals (mice received 1 injection/day of 5 ug of IL-1- $\alpha$ ); Low dose muOPG [22-401]-Fc treated animals (mice received 1 injection/day of 10  $\mu$ g of muOPG [22-401]-Fc; Low dose muopg [22-401]-Fc and IL-1- $\alpha$ , (dosing as above); High dose muopg [22-401]-Fc treated animals (mice received 3 injections/day of 10  $\mu$ g muOPG [22-401]-Fc; High dose muOPG [22-401]-Fc and IL-1- $\alpha$ . All mice received the same number of injections/day of either active factor or vehicle. All groups were sacrificed on the day after the last injection. The blood ionized calcium levels were measured before the first injection, four hours after the second injection and 24 hours after the third IL-1 injection, just before the animals were sacrificed. The animal weights were measured before the first injection, four hours after the second injection and 24 hours after the third IL-1 injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin sectioning.

Histological methods

Calvarial bone samples were fixed in zinc formalin, decalcified in formic acid, dehydrated through ethanol and mounted in paraffin. Sections (5 $\mu$ m thick) were cut through the calvaria adjacent to the lambdoid suture and stained with either hematoxylin and eosin or reacted for tartrate resistant acid phosphatase activity (Sigma Kit# 387A) and counterstained with hematoxylin. Bone resorption was assessed in the IL-1  $\alpha$  treated mice by histomorphometric methods using the Osteomeasure (Osteometrics, Atlanta, GA) by tracing histologic features onto a digitizer platen using a microscope-mounted camera lucida attachment. Osteoclast numbers, osteoclast lined surfaces, and eroded surfaces were determined in the marrow spaces of the calvarial bone. The injected and non-injected sides of the calvaria were measured separately.

Results

IL-1  $\alpha$  and IL-1  $\beta$  produced hypercalcemia at the doses used, particularly on the second day, presumably by the induction of increased bone resorption systemically. The hypercalcemic response was blocked by muOPG [22-401]-Fc in the IL-1 beta treated mice and significantly diminished in mice treated with IL-1- $\alpha$ , an effect most apparent on day 2 (Figure 22A-22B).

Histologic analysis of the calvariae of mice treated with IL-1- $\alpha$  and beta shows that IL-1 treatments alone produce a marked increase in the indices of bone resorption including: osteoclast number, osteoclast lined surface, and eroded surface (surfaces showing deep scalloping due to osteoclastic action (Figure 23B)). In response to IL-1  $\alpha$  or IL-1  $\beta$ , the increases in bone resorption were similar on the injected and non-

injected sides of the calvaria. Muopg [22-401]-Fc injections reduced bone resorption in both IL-1- $\alpha$  and beta treated mice and in mice receiving vehicle alone but this reduction was seen only on the muopg [22-401]-Fc injected sides of the calvariae.

The most likely explanation for these observations is that muOPG [22-401]-Fc inhibited bone resorption, a conclusion supported by the reduction of both the total osteoclast number and the percentage of available bone surface undergoing bone resorption, in the region of the calvaria adjacent to the muOPG [22-401]-Fc injection sites. The actions of muOPG [22-401]-Fc appeared to be most marked locally by histology, but the fact that muOPG [22-401]-Fc also blunted IL-1 induced hypercalcemia suggests that muOPG [22-401]-Fc has more subtle effects on bone resorption systemically.

C. Systemic Effects of muOPG [22-401]-Fc in Growing Mice

Male BDF1 mice aged 3-4 weeks, weight range 9.2-15.7g were divided into groups of ten mice per group. These mice were injected subcutaneously with saline or muOPG [22-401]-Fc 2.5mg/kg bid for 14 days (5mg/kg/day). The mice were radiographed before treatment, at day 7 and on day 14. The mice were sacrificed 24 hours after the final injection. The right femur was removed, fixed in zinc formalin, decalcified in formic acid and embedded in paraffin. Sections were cut through the mid region of the distal femoral metaphysis and the femoral shaft. Bone density, by histomorphometry, was determined in six adjacent regions extending from the metaphyseal limit of the growth plate, through the primary and secondary spongiosa and into the femoral diaphysis (shaft). Each region was 0.5 x 0.5 mm<sup>2</sup>.

Radiographic changes

After seven days of treatment there was evidence of a zone of increased bone density in the spongiosa associated with the growth plates in the OPG treated 5 mice relative to that seen in the controls. The effects were particularly striking in the distal femoral and the proximal tibial metaphyses (Figure 24A-24B). However bands of increased density were also apparent in the vertebral bodies, the iliac crest and the distal 10 tibia. At 14 days, the regions of opacity had extended further into the femoral and tibial shafts though the intensity of the radio-opacity was diminished. Additionally, there were no differences in the length 15 of the femurs at the completion of the experiment or in the change in length over the duration of the experiment implying that OPG does not alter bone growth.

Histological Changes

The distal femoral metaphysis showed increased 20 bone density in a regions 1.1 to 2.65 mm in distance from the growth plate (Figures 25 and 26A-26B). This is a region where bone is rapidly removed by osteoclast-mediated bone resorption in mice. In these rapidly growing young mice, the increase in bone in this region 25 observed with OPG treatment is consistent with an inhibition of bone resorption.

D. Effects of Osteoprotegerin on Bone Loss Induced by Ovariectomy in the Rat

Twelve week old female Fisher rats were 30 ovariectomized (OVX) or sham operated and dual xray absorptiometry (DEXA) measurements made of the bone density in the distal femoral metaphysis. After 3 days recovery period, the animals received daily injections for 14 days as follows: Ten sham operated animals 35 received vehicle (phosphate buffered saline); Ten OVX animals received vehicle (phosphate buffered saline);

Six OVX animals received OPG-Fc 5mg/kg SC; Six OVX animals received pamidronate (PAM) 5mg/kg SC; Six OVX animals received estrogen (ESTR) 40ug/kg SC. After 7 and 14 days treatment the animals had bone density measured by DEXA. Two days after the last injection the animals were killed and the right tibia and femur removed for histological evaluation.

The DEXA measurements of bone density showed a trend to reduction in the bone density following ovariectomy that was blocked by OPG-Fc. Its effects were similar to the known antiresorptive agents estrogen and pamidronate. (Figure 27). The histomorphometric analysis confirmed these observations with OPG-Fc treatment producing a bone density that was significantly higher in OVX rats than that seen in untreated OVX rats (Figure 28). These results confirm the activity of OPG in the bone loss associated with withdrawal of endogenous estrogen following ovariectomy.

20        In vivo Summary

The in vivo actions of recombinant OPG parallel the changes seen in OPG transgenic mice. The reduction in osteoclast number seen in the OPG transgenic is reproduced by injecting recombinant OPG locally over the calvaria in both normal mice and in mice treated with IL-1  $\alpha$  or IL-1  $\beta$ . The OPG transgenic mice develop an osteopetrotic phenotype with progressive filling of the marrow cavity with bone and unremodelled cartilage extending from the growth plates from day 1 onward after birth. In normal three week old (growing) mice, OPG treatments also led to retention of bone and unremodelled cartilage in regions of endochondral bone formation, an effect observed radiographically and confirmed histologically. Thus, recombinant OPG produces phenotypic changes in normal animals similar

to those seen in the transgenic animals and the changes are consistent with OPG-induced inhibition of bone resorption. Based on *in vitro* assays of osteoclast formation, a significant portion of this inhibition is 5 due to impaired osteoclast formation. Consistent with this hypothesis, OPG blocks ovariectomy-induced osteoporosis in rat. Bone loss in this model is known to be mediated by activated osteoclasts, suggesting a role for OPG in treatment of primary osteoporosis.

10

**EXAMPLE 12****Pegylation Derivatives of OPG****Preparation of N-terminal PEG-OPG conjugates by reductive alkylation**

HuOPG met [22-194] P25A was buffer exchanged into 15 25-50 mM NaOAc, pH 4.5-4.8 and concentrated to 2-5 mg/ml. This solution was used to conduct OPG reductive alkylation with monofunctional PEG aldehydes at 5-7 C. PEG monofunctional aldehydes, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were 20 added to the OPG solution as solids in amounts constituting 2-4 moles of PEG aldehyde per mole of OPG. After dissolution of polymer into the protein solution, sodium cyanoborohydride was added to give a final concentration of 15 to 20 mM in the reaction mixture 25 from 1-1.6 M freshly prepared stock solution in cold DI water. The progress of the reaction and the extent of OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the 30 reaction was allowed to proceed for 16-18 hours, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 35 with a linear gradient to 0.75M NaCl over 25 column

volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE. By N-terminal sequencing, it was determined that the monoPEG-OPG conjugate, the major reaction product in most cases, was 98% N-terminally PEG-modified OPG.

This procedure was generally used to prepare the following N-terminal PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A: 5 kD monoPEG, 10 kD mono branched PEG, 12 kD monoPEG, 20 kD monoPEG, 20 kD mono branched PEG, 25 kD monoPEG, 31 kD monoPEG, 57 kD monoPEG, 12 kD diPEG, 25 kD diPEG, 31 kD diPEG, 57 kD diPEG, 25 kD triPEG).

Preparation of PEG-OPG conjugates by acylation

HuOPG met [22-194] P25A was buffer exchanged into 50 mM BICINE buffer, pH 8 and concentrated to 2-3 mg/ml. This solution was used to conduct OPG acylation with monofunctional PEG N-hydroxysuccinimidyl esters at room temperature. PEG N-hydroxysuccinimidyl esters, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 4-8 moles of PEG N-hydroxysuccinimidyl ester per mole of OPG. The progress of the reaction and the extent of OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 1 hour, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE.

This procedure was generally used to prepare the following PEG-OPG conjugates: 5 kD polyPEG, 20 kD polyPEG, 40 kD poly branched PEG, 50 kD poly PEG.

Preparation of dimeric PEG-OPG

- 5        HuOPG met [22-194] P25A is prepared for thiolation at 1-3 mg/ml in a phosphate buffer at near neutral pH. S-acetyl mecaptosuccinic anhydride (AMSA) is added in a 3-7 fold molar excess while maintaining pH at 7.0 and the rxn stirred at 4°C for 2 hrs. The monothiolated-OPG  
10      is separated from unmodified and polythiolated OPG by ion exchange chromatography and the protected thiol deprotected by treatment with hydroxylamine. After deprotection, the hydroxylamine is removed by gel filtration and the resultant monothiolated-OPG is  
15      subjected to a variety of thiol specific crosslinking chemistries. To generate a disulfide bonded dimer, the thiolated OPG at >1mg/ml is allowed to undergo air oxidation by dialysis in slightly basic phosphate buffer. The covalent thioether OPG dimer was prepared  
20      by reacting the bis-maleimide crosslinker, N,N-bis(3-maleimidopropyl)-2-hydroxy 1,3 propane with the thiolated OPG at >1mg/ml at a 0.6x molar ratio of crosslinker:OPG in phosphate buffer at pH 6.5.  
Similarly, the PEG dumbbells are produced by reaction  
25      of substoichiometric amounts of bis-maleimide PEG crosslinkers with thiolated OPG at >1mg/ml in phosphate buffer at pH 6.5. Any of the above dimeric conjugates may be further purified using either ion exchange or size exclusion chromatographies.  
30      Dimeric PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A prepared using the above procedures include disulfide-bonded OPG dimer, covalent thioether OPG dimer with an aliphatic amine type crosslinker, 3.4 kD and 8kD PEG dumbbells and monobells.

PEG-OPG conjugates were tested for activity in vitro using the osteoclast maturation assay described in Example 11A and for activity in vivo by measuring increased bone density after injection into mice as 5 described in Example 11C. The in vivo activity is shown below in Table 2.

Table 2In vivo biological activity of PEGylated OPG

10	<u>OPG Construct</u>	<u>Increase in Tibial Bone Density</u>
----	----------------------	--

	muOPG met [22-194]	-
	muOPG met [22-194] 5k PEG	+
	muOPG met [22-194] 20k PEG	+
15		
	huOPG met [22-194] P25A	-
	huOPG met [22-194] P25A 5k PEG	+
	huOPG met [22-194] P25A 20k PEG	+
	huOPG met [22-194] P25A 31k PEG	+
20	huOPG met [22-194] P25A 57k PEG	+
	huOPG met [22-194] P25A 12k PEG	+
	huOPG met [22-194] P25A 20k Branched PEG	+
	huOPG met [22-194] P25A 8k PEG dimer	+
	<u>huOPG met [22-194] P25A disulfide crosslink</u>	+

25	<u>EXAMPLE 13</u>
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<u>Effects of OPG-Fc during the course of Adjuvant</u>
<u>Arthritis in Lewis rats</u>

The aim of these studies is to investigate whether CHO produced OPG-Fc protects against adjuvant 30 arthritis-associated bone mineral density loss in male Lewis rats.

Animals

Male Lewis rats (Charles River, Wilmington MA) 8-9 weeks of age (n = 6) at the time of mycobacteria in oil 35 injection, were used. Two rats were housed per cage in an air conditioned environment (room temperature 23 ± 2

- C, relative humidity 50 ± 20%) that illuminated from 6:30 am to 6:30 p.m. Animals were fed a commercial rodent chow (#8640, Tek Lab, Madison WI); calcium and phosphorus contents were 1.2% and 1.0%, respectively.
- 5 All animals were sacrificed by carbon dioxide inhalation.

Induction and Measurement of Adjuvant Arthritis

Adjuvant arthritis (AdA) was induced by a single injection of a suspension of Mycobacterium tuberculosis (Difco Laboratories, Detroit MI) in paraffin oil (Crescent Chemical Co., Hauppauge, NY). Mycobacteria were ground in a mortar to fine powder and suspended in paraffin oil (10 mg/ml). The suspension was dispersed evenly just before injection of 0.05ml at the base of tail. Severity of inflammation was monitored by measuring the volume of hindpaws using volume displacement technique. The extent of inflammation was calculated as increase in paw volume compared to Day 0. In addition, body weight was measured daily.

20 OPG treatment and DEXA bone mass measurement

Male Lewis (normal and adjuvant-induced) rats received varying doses of OPG-Fc (22-194) by subcutaneous daily injection (See graphs below for dosing) from day 9 to day 15. At the end of the 25 experiment (day 16) bone mass measurement (DexaScans) of the tibiotarsal region was performed with a Hologic QDR 4500 dual-energy x-ray absorptiometer.

Statistical Analysis

All results were expressed as the mean ± standard deviation of the mean. The p value of 0.05 was used in the calculation to determine whether there were any significant differences between any two groups. Statistical significance of difference was assessed by analysis of variance based on a Mann Whitney U test 35 using Statsoft software (Statsoft, Tulsa, OK).

ResultsOPG-Fc inhibits loss of Bone Mineral Density in adjuvant arthritis

To study the effects of OPG-Fc on BMD in adjuvant 5 arthritis, paws from two experiments were analyzed by DEXA. The results of BMD measurements on the tibiotarsal region are shown in Figures 2 and 4. Bone protective effects were observed in rats with adjuvant- arthritis treated with OPG-Fc via subcutaneous daily 10 injection (from day 9 to day 15 after mycobacteria injection). Treatment with OPG-Fc at 4, 1, 0.25, 0.06, .016, and 0.004 mg/kg showed 100%, 100%, 100%, 86%, 22, and 22% inhibition of bone mineral density loss respectively. Treatment of the intermediate and high 15 doses of OPG-Fc (4 - 0.06 mg/kg) showed a statistically significant difference in BMD when compared to the OPG placebo treated control group ( $P < 0.05$ ).

However, treatment with OPG-Fc (at all doses) had no statistically significant effect on the severity of 20 inflammation (Figure 1 and 3, AUC) or loss of body weight (data on file).

Conclusion

In conclusion, the results demonstrate that OPG-Fc have great efficacy in preventing bone density loss in 25 the tibiotarsal region in arthritic rats. The inhibitory effects of OPG-Fc against bone changes occurred without any anti-inflammatory actions.

EXAMPLE 14Combination treatment with OPG-Fc and sTNF-RI on Adjuvant Arthritis in Male Lewis Rats

Male Lewis rats were injected with 0.5 mg heat-killed Mycobacterium tuberculosis H37Ra in mineral oil at the base of the tail. Rats were monitored for paw swelling and weight loss. Arthritis (paw swelling) 35 developed after about 10 days. Paw swelling was calculated daily relative to paw volume on day 9

(beginning of treatment) and the area under the curve (AUC) from day 9 to 15 is given in the graph (Figure 31A). On day 16 at the end of the experiment DexaScans of the rats were taken and the calcaneus was evaluated 5 for loss of bone mineral density (BMD) as shown in Figure 31B.

\* \* \*

While the invention has been described in what is considered to be its preferred embodiments, it is not 10 to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such 15 modifications and equivalents.

Claims

What is claimed is:

1. A method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with substances selected from the group consisting of TNF- $\alpha$  inhibitors; serine protease inhibitors; IL-1 inhibitors; IL-6 inhibitors; IL-8 inhibitors; IL-18 inhibitors; ICE modulators; FGF-1 to FGF-10; FGF modulators; PAF antagonists; MMP modulators; NOS modulators; modulators of glucocorticoid receptor; modulators of glutamate receptor; modulators of LPS levels; and noradrenaline and modulators and mimetics thereof.
2. The method of Claim 1, wherein the OPG protein is OPG-Fc.
3. The method of claim 1, wherein an IL-1 inhibitor and the OPG protein are administered.
4. The method of claim 3, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
5. The method of Claim 4, wherein the OPG protein comprises an Fc region.
6. The method of claim 1, wherein a TNF- $\alpha$  inhibitor and the OPG protein are administered.
7. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, or a fragment of STNF-RI or STNF-RII linked to an Fc region.
8. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG sTNFR-I.
9. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises a 2.6 kD sTNF-RI fragment.
10. The method of claim 9, wherein the sTNF-RI fragment comprises 30 kD PEG.

11. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises sTNF-RII linked to an Fc region.
12. The method of claim 6, wherein the TNF- $\alpha$  inhibitor is etanercept.
13. The method of Claim 10, wherein the OPG protein is OPG-Fc.
14. The method of claim 1, wherein a serine protease inhibitor and the OPG protein are administered.
15. The method of claim 14, wherein the serine protease inhibitor comprises a SLPI polypeptide.
16. The method of Claim 14, wherein the OPG protein is OPG-Fc.
17. The method of claim 1, wherein an IL-1 inhibitor, a TNF- $\alpha$  inhibitor, and the OPG protein are administered.
18. The method of claim 17, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
19. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR-Fc.
20. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG-sTNFR-I.
21. The method of claim 17, wherein the sTNF-RI fragment is a 2.6 kD fragment.
22. The method of claim 21, wherein the sTNF-RI fragment comprises 30 kD PEG.
23. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-II linked to an Fc region.
24. The method of claim 17, wherein the TNF- $\alpha$  inhibitor is etanercept.
25. The method of Claim 17, wherein the OPG protein is OPG-Fc.

26. The method of Claim 1, wherein an IL-1 inhibitor, a serine protease inhibitor, and the OPG protein are administered.
27. The method of claim 26, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
28. The method of claim 26, wherein the serine protease inhibitor comprises a SLPI polypeptide.
29. The method of Claim 26, wherein the OPG protein is OPG-FC.
30. The method of claim 1, wherein a serine protease inhibitor, a TNF- $\alpha$  inhibitor, and the OPG protein are administered.
31. The method of claim 30, wherein the serine protease inhibitor comprises a SLPI polypeptide.
32. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR-FC.
33. The method of claim 30, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG sTNFR-I.
34. The method of claim 30, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-II linked to an Fc region.
35. The method of claim 30, wherein the TNF- $\alpha$  inhibitor is etanercept.
36. The method of claim 30, wherein the TNF- $\alpha$  inhibitor comprises a 2.6 kD sTNF-RI fragment.
37. The method of claim 36, wherein the sTNF-RI fragment comprises 30 kD PEG.
38. The method of Claim 30, wherein the OPG protein is OPG-FC.
39. The method of any of claims 17 to 38, wherein the condition treated is rheumatoid arthritis.
40. The method of any of claims 17 to 38, wherein the condition treated is multiple sclerosis.

41. The method of any of claims 17 to 38, wherein the condition treated is osteoporosis.
42. The method of any of claims 17 to 38, wherein the condition treated is osteomyelitis.
43. A method of treating an IL-1 mediated disease, which comprises administering therapeutically effective amounts of an IL-1 inhibitor and a serine protease inhibitor.
44. The method of claim 43, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
45. The method of claim 43, wherein the serine protease inhibitor comprises a SLPI polypeptide.
46. The method of claim 43, wherein the IL-1 mediated disease is asthma.
47. The method of claim 43, wherein the IL-1 mediated disease is rheumatoid arthritis.
48. The method of claim 46, wherein the therapeutically effective amounts are delivered by pulmonary administration.
49. A method of treating TNF-mediated disease, which comprises administering therapeutically effective amounts of a TNF- $\alpha$  inhibitor and a serine protease inhibitor.
50. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR-Fc.
51. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG sTNFR-I.
52. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises a 2.6 kD sTNF-RI fragment.
53. The method of claim 52, wherein the sTNF-RI fragment comprises 30 kD PEG.

54. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-II linked to an Fc region.
55. The method of claim 49, wherein the TNF- $\alpha$  inhibitor is etanercept.
56. The method of claim 49, wherein the serine protease inhibitor comprises a SLPI polypeptide.
57. The method of claim 49, wherein the TNF-mediated disease is rheumatoid arthritis.
58. A method of treating inflammation, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.
59. A method of treating rheumatoid arthritis, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.
60. A method of treating SLE, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.
61. A method of treating GvHD, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.

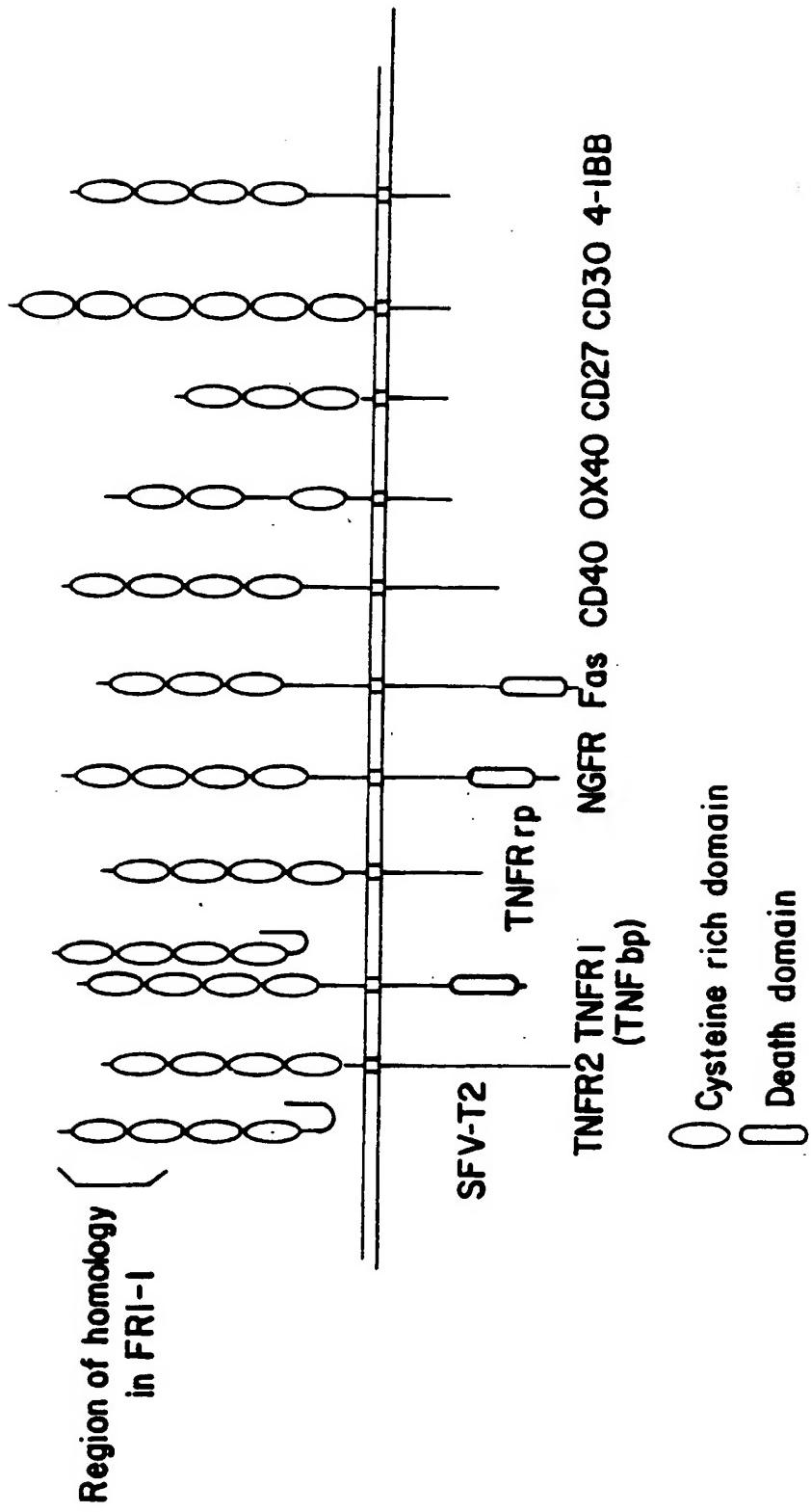
**FIG. 1A**

<b>FRI-1</b> <b>SW: TNR2_HUMAN</b>	148      178      208      238      268      298 ALLVFLDIEWTTQETFPKYLHYDPETrQLLCDKCAPGTYLQHCTVRRKTLCVPCPD I :       :     :           :   :     HALPAQVAFTPYAPEPGSTCRLEYYDQTAQMCCSKCSPGQHAKVFCTKTSDTVCDSCED 30      40      50      60      70      80
<b>FRI-1</b> <b>SW: TNR2_HUMAN</b>	328 YSYTDSWHTS :     :   : STYTQLWNWVPECLSGSSRCSSDQVETQACTREQNRICTCRPGWYCALSKQEGCRLCABL 90      100      110      120      130      140

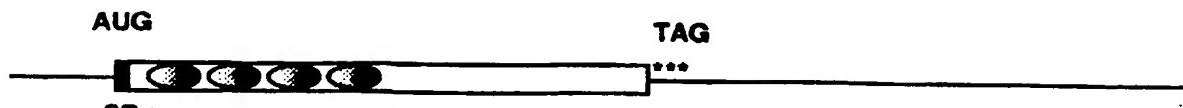
**FIG. 1B**

<b>FRI-1</b> <b>TNFR profile</b>	69    YLHYDPETrGRQLLCDKCAPGTYLQHCTVRRKTLCV.PCPDY.SYTDSSW     . . . .     :   .   :       :   .   . 6    YHYDQNGRMCEECHMCQPGHFLVKHCKQPKRDTVCHKPCEPGVTYTTDDW
<b>FRI-1</b> <b>TNFR profile</b>	116 H   56 H Z Score = 8.29

FIG. IC



## FIG.2A



## FIG.2B

10	30	50
ATCAAAGGCAGGGCATACTTCCTGTTGCCAGACCTTATATAAAACGTCA	TGTTCCGCTG	
70	90	110
GGCAGCAGAGAACGACCTAGCACTGGCCCAGCGGCTGCCGCTGAGGTTCCAGAGGACC		
130	150	170
ACAATGAACAAGTGGCTGTGCTGTGACTCCTGGTGTCTTGACATCATTGAATGGACA		
M N K W L C C A L L V F L D I I E W T		
190	210	230
ACCCAGGAAACCTTCCCTCCAAAATACCTGCATTATGACCCAGAAACCGGACGTCAGCTC		
T Q E T F P P K Y L H Y D P E T G R Q L		
250	270	290
TTGTGTGACAAATGTGCTCCTGGCACCTACCTAAACAGCACTGCACAGTCAGGAGGAAG		
L C D K C A P G T Y L K Q H C T V R R K		
310	330	350
ACACTGTGTGTCCTGCCCTGACTACTCTTATACAGACAGCTGGCACACGAGTGATGAA		
T L C V P C P D Y S Y T D S W H T S D E		
370	390	410
TGCGTGTACTGCAGCCCCGTGTGCAAGGAACTGCAGACCGTGAAACAGGAGTGCAACCGC		
C V Y C S P V C K E L Q T V K Q E C N R		
430	450	470
ACCCACAAACCGAGTGTGCGAATGTGAGGAAGGGCGCTACCTGGAGCTCGAATTCTGCTTG		
T H N R V C E C E E G R Y L E L E F C L		
490	510	530
AAGCACCGGAGCTGTCCCCCAGGCTGGGTGTGCTGCAGGCTGGGACCCAGAGCGAAC		
K H R S C P P G L G V L Q A G T P E R N		
550	570	590
ACGGTTTGCACAAAGATGTCCGGATGGGTTCTCTCAGGTGAGACGTATCGAAAGCACC		
T V C K R C P D G F F S G E T S S K A P		
610	630	650
TGTAGGAAACACACCAACTGCAGCTCACTGGCCTCTGCTAATTCAAGAAAGGAAATGCA		
C R K H T M C S S L G L L I Q K G N A		
670	690	710
ACACATGACAATGTATGTTCCGAAACAGAGAAGCAACTCAAATTGTGGAATAGATGTC		
T H D N V C S G N R E A T Q N C G I D V		
730	750	770
ACCTGTGCGAACAGGGCATTCTCAGGTTGCTGTGCCCTACCAAGATTATACCGAATTGG		
T L C E E A F F R F A V P T K I I P N W		
790	810	830
CTGAGTGTCTGGGGACAGTTGCCCTGGGACCAAAAGTGAATGCAGAGAGTGTAGAGAGG		
L S V L V D S L P G T K V N A E S V E R		
850	870	890
ATAAAACGGAGACACAGCTCGCAAGAGCAAACCTTCAGCTACTTAAGCTGTGAAAGCAT		
I K R R H S S Q E Q T F Q L L K L W K H		
910	930	950
CAAAACAGAGAACAGGAAATGGTGAAGAAGATCATCCAAGACATTGACCTCTGTGAAAGC		
Q N R D Q E M V K K I I Q D I D L C E S		
970	990	1010
AGTGTGCAACGGCATATCGGCCACCGAACCTCACCAAGACATTGACCTCTGTGAAAGC		
S V Q R H I G H A N L T T E Q L R I L M		

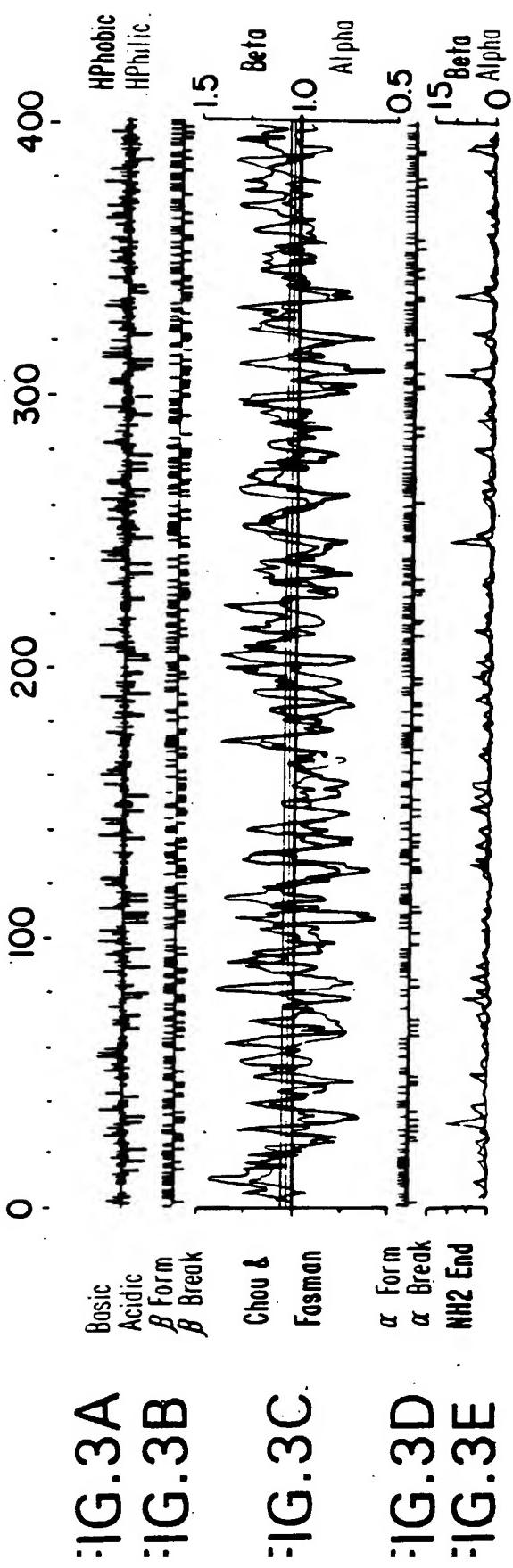
## FIG.2C

1030	1050	1070
GAGAGCTTGCCTGGAAAGAAGATCAGCCCAGACGAGATTGAGAGAACGAGAAAGACCTGC		
E S L P G K K I S P D E I E R T R K T C		
1090	1110	1130
AAACCCAGCGAGCAGCTCCTGAAGCTACTGAGCTTGTGGAGGATCAAAAATGGAGACCAA		
K P S E Q L L K L L S L W R I K N G D Q		
1150	1170	1190
GACACCTGAAGGGCTGATGTACGCAGCTCAAGCACTTGAAAGCATACCACTTCCAAA		
D T L K G L M Y A L K H L K A Y H F P K		
1210	1230	1250
ACCGTCACCCACAGTCTGAGGAAGACCATTAGGTTCTGCACAGCTTACCATGTACCGA		
T V T H S L R K T I R F L H S F T M Y R		
1270	1290	1310
TTGTATCAGAAACTCTTCTAGAAATGATAGGAATCAGGTTCAATCAGTGAAGATAAGC		
L Y Q K L F L E M I G N Q V Q S V K I S		
1330	1350	1370
TGCTTATAGTTAGGAATGGCACTGGCTTTCTCAGGATGGCCAACACTGATGGAG		
C L		
1390	1410	1430
CAGATGGCTGCTTCTCCGGCTTGAAATGGCAGTTGATTCTTCTCATCAGTTGGTGG		
1450	1470	1490
GAATGAAGATCCTCCAGCCAACACACACACTGGGGAGTCTGAGTCAGGAGAGTGAGGCA		
1510	1530	1550
GCGTATTGATAATTGTGCCAAAGCTGCCAGGTGTACACCTAGAAAGTCAAAGCACCGTGAG		
1570	1590	1610
1630	1650	1670
TAATCAGAAGGCTTCTACTATCTTCTGTGTCATCCCTAGATGAAGGCCTTTTATTTAT		
1690	1710	1730
TTTTTTATTCTTTTTTCGGAGCTGGGGACCGAACCCAGGGCTTGCCTGCGAGGCAA		
1750	1770	1790
GTGCTCTACCACTGAGCTAAATCTCAAACCCCTGAAGGCCTTTCTGCCTCTGAT		
1810	1830	1850
AGTCTATGACATTCTTTCTACAATTGTGTCAGGTGCACGAGCCTTATCCCATTGT		
1870	1890	1910
AGGTTCTAGGCAAGTTGACCGTTAGCTATTTCCCTCTGAAGATTGATTGAGTTGC		
1930	1950	1970
AGACTTGGCTAGACAAGCAGGGTAGGTTATGGTAGTTATTAACAGACTGCCACCAGG		
1990	2010	2030
AGTCCAGTCTTCTGTTCCCTGTAGTTGACCTAACGCTGACTCCAAGTACATTAGTA		
2050	2070	2090
TGAAAAATLATCAACAAATTATTCTTCTATCAACATTGGCTAGCTTGTGAGGGC		
2110	2130	2150
ACTAAAAAGAAACTACTATATGGAGAAAGAATTGATATTGCCCAACGTTCAACAAACCA		
2170	2190	2210
ATAGTTTATCCAGCTGTGTCATGCCCTGGTTAGTGTCTACTGACTATGCCCTTATTAC		
2230	2250	2270
TGCATGCAGTAATTCAACTGGAAATAGTAATAATAATAGAAATAAAATCTAGACTCC		
2290	2310	2330
ATTGGATCTCTGAATATGGGAATATCTAACTTAAGAAGCTTGAGATTCAAGTGTGT		
2350	2370	2390
TAAAGGCTTTATTAAAAAGCTGATGCTCTGTAAAAGTTACTAATATCTGTAAGA		
2410	2430	

FIG. 2D

fas.frg	H F -	- S S K C R R -	- S S K C R K E M G Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F Q C F	103
enfr1.frg	H L R H C L S C V S C R G -	- A P A C L S C G S -	- V P E C L S C E P -	144
fv-t2.frg	H -	- A P A C L S C V S C R G -	- V P E C L S C E P -	84
enfr2.frg	H -	- V P E C L S C V S C R G -	- H R H C L S C E P -	98
cd40.frg	R E I R C H Q H R H C L S C V S C R G -	- T S D E C V Y C S P -	- C V Y C S P -	85
osteо.frg	-	- T S D E C V Y C S P -	- C V Y C S P -	65
ngfr.frg	A T E P C K P C T E C -	- C V Y C S P -	- C V Y C S P -	89
ox40.frg	- Y D T C K Q C T Q C N H -	- C V Y C S P -	- C V Y C S P -	84
4lbb.frg	- I G G Q P N C N T C R -	- C V Y C S P -	- C V Y C S P -	65

**FIG. 2E**



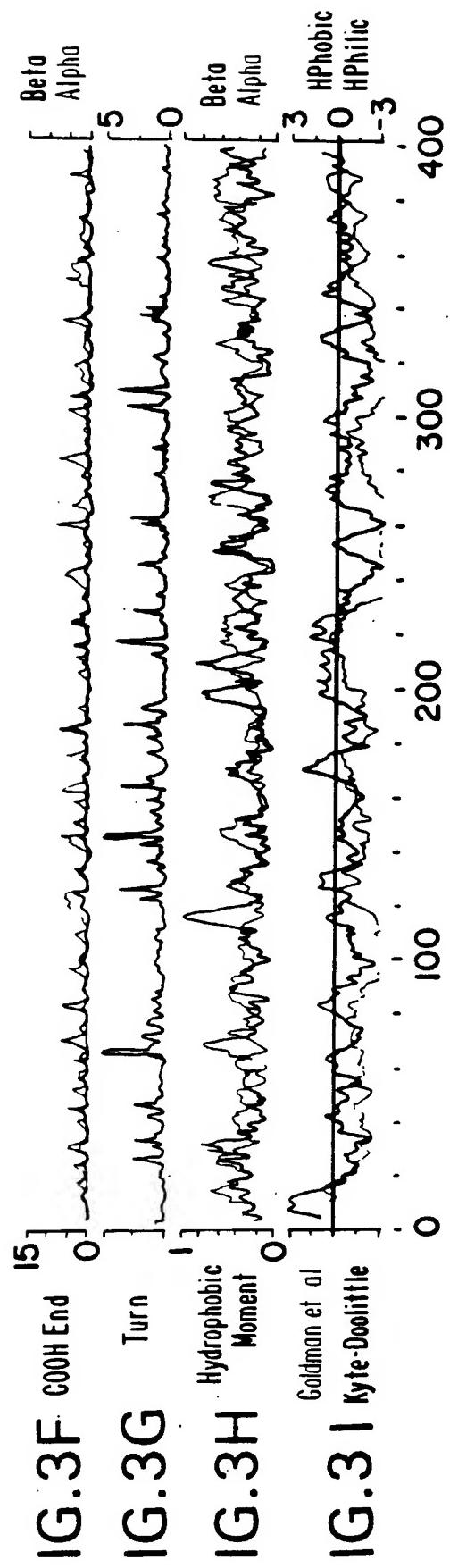


FIG.4A

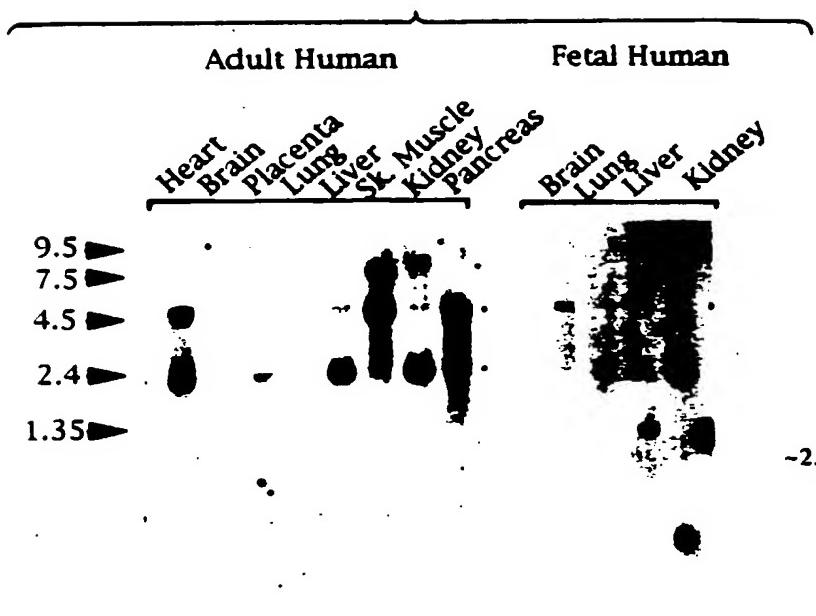
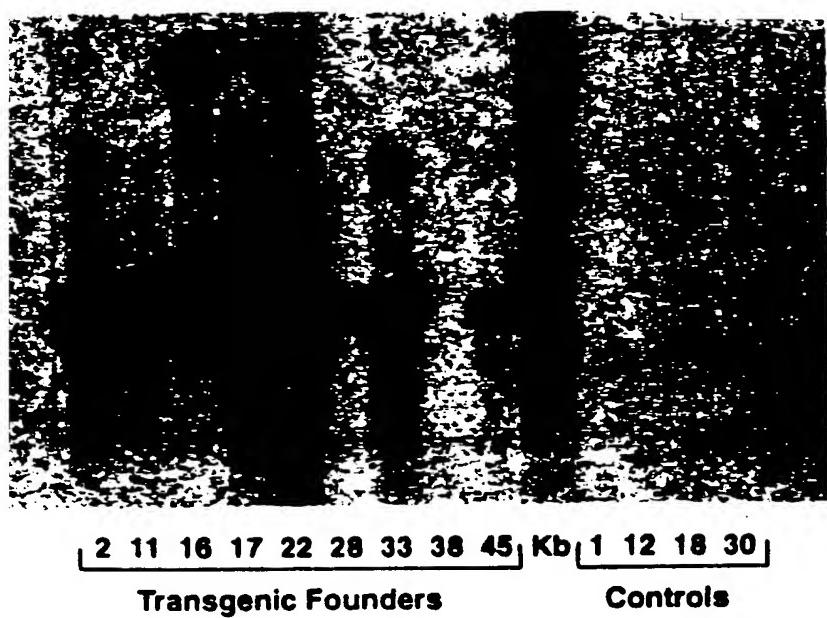


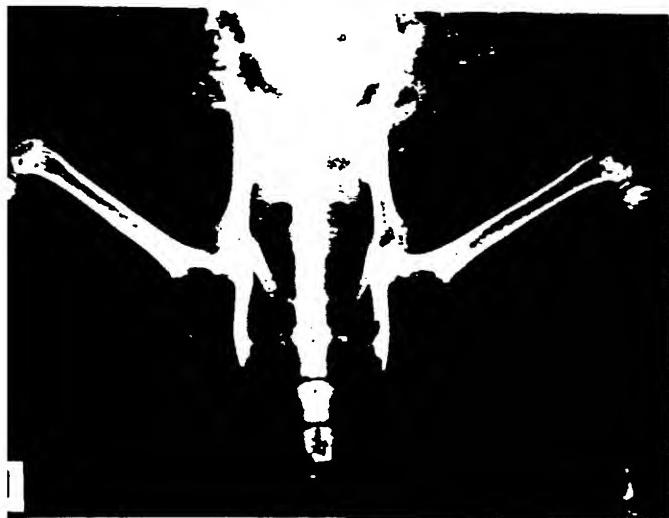
FIG. 4B

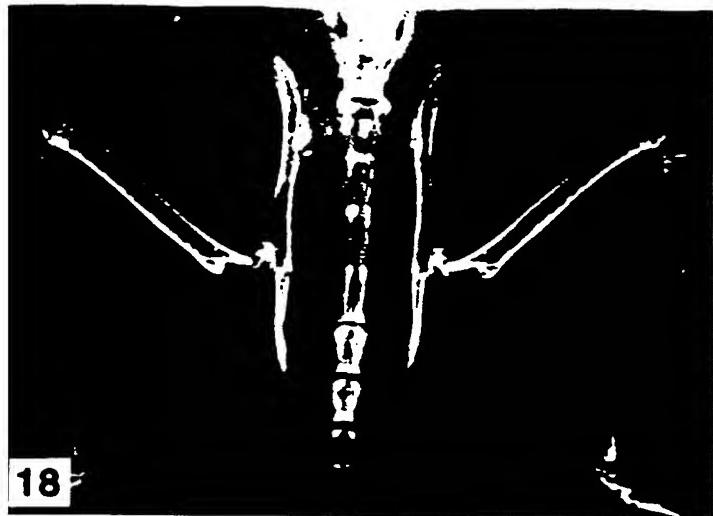
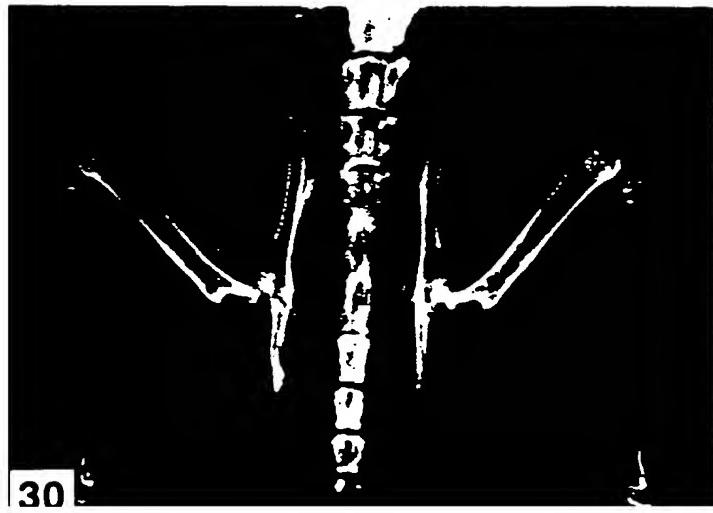
## **Human Immune System**

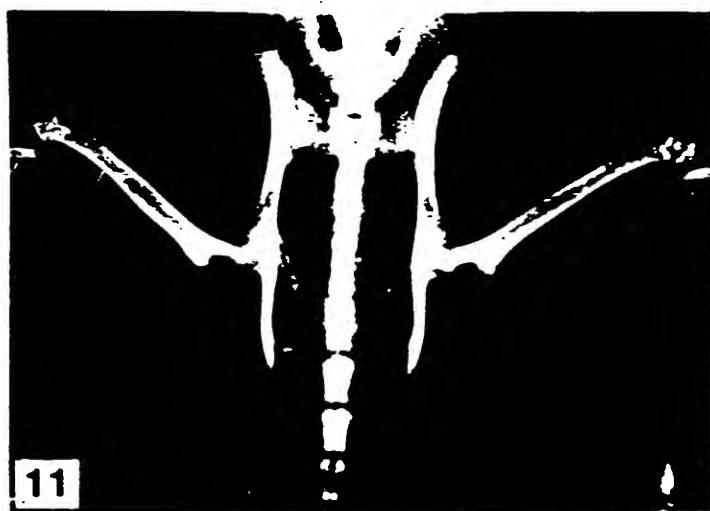


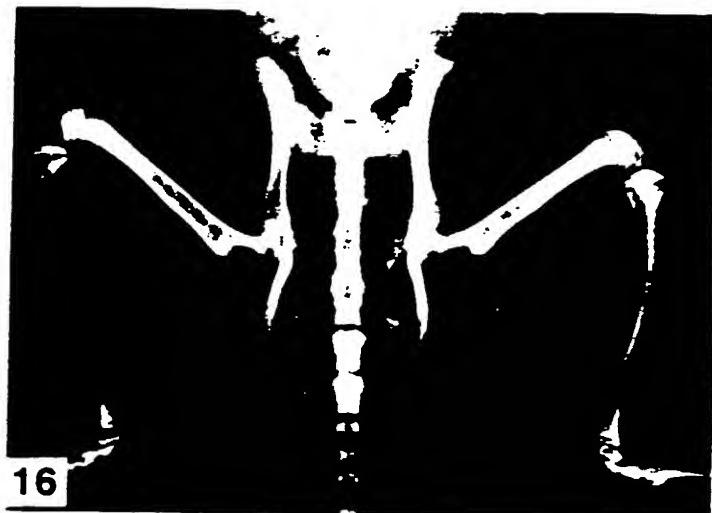
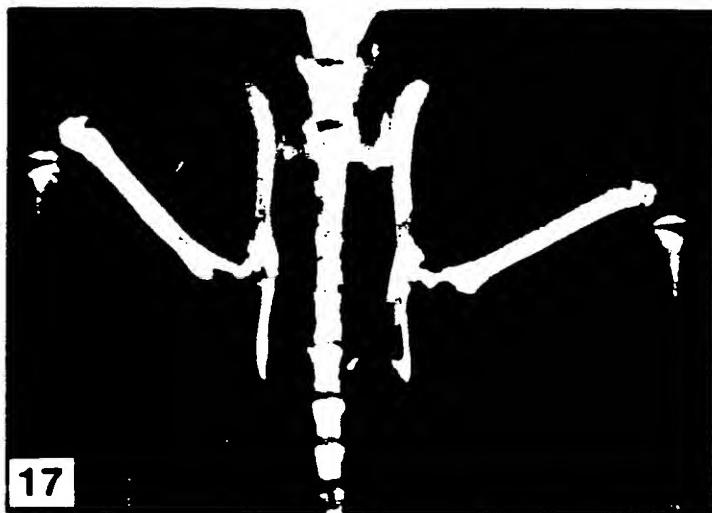
**FIG.5**

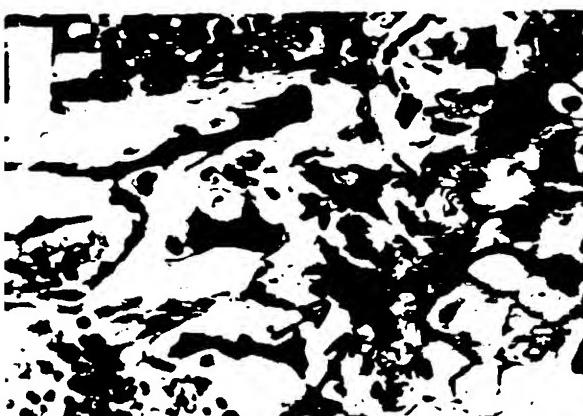


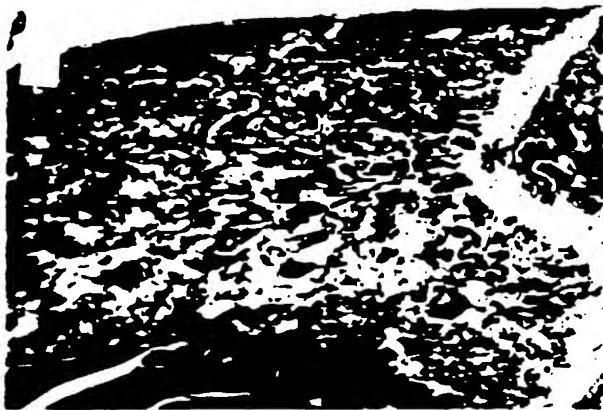
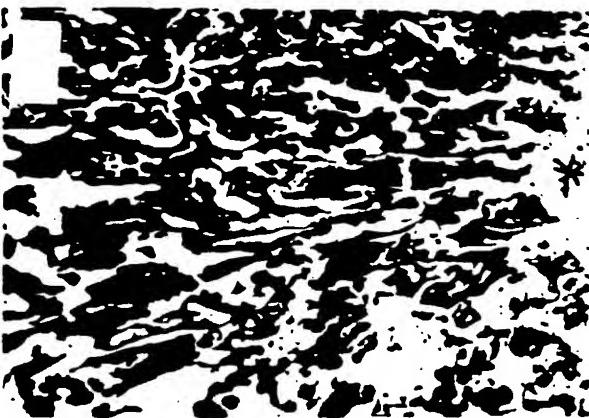
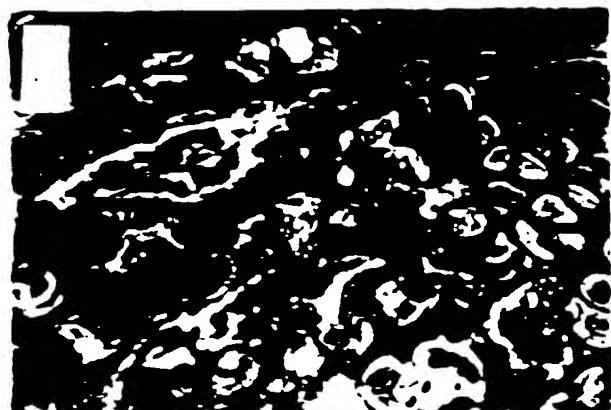
**FIG.6A****FIG.6B****FIG.6C**

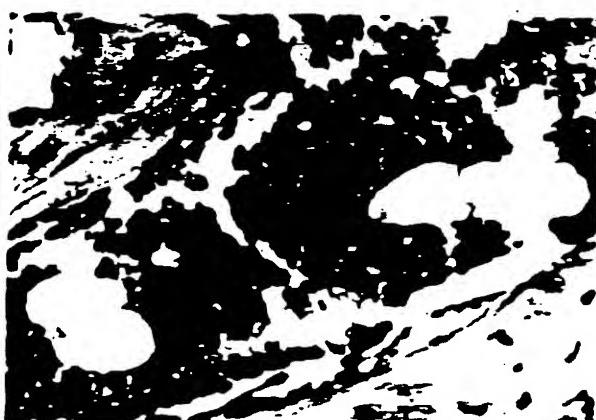
**FIG.6D****FIG.6E****FIG.6F**

**FIG.6G****FIG.6H**

**FIG.6I****FIG.6J**

**FIG.7A****FIG.7B****FIG.7C****FIG.7D**

**FIG. 7E****FIG. 7F****FIG. 7G****FIG. 7H**

**FIG.8A****FIG.8B****FIG.8C****FIG.8D**

**FIG.9A**

# FIG. 9B

790	810	830
AAAGTGAATGCCGAGAGTGAGAGAGGATAAAACGGAGACACAGCTCACAAAGAGCAAACC		
K V N A E S V E R I K R R H S S Q E Q T		
850	870	890
TTCCAGCTGCTGAAGCTGTGGAAACATCAAAACAGAGACCAGGAAATGGTGAAGAAGATC		
F Q L L K L W K H Q N R D Q E M V K K I		
910	930	950
ATCCAAGACATTGACCTCTGTGAAAGCAGCGTGCAGCGGCATCTCGGCCACTCGAACCTC		
I Q D I D L C E S S V Q R H L G H S N L		
970	990	1010
ACCACAGAGCAGCTTCTTGCCTTGATGGAGAGCCTGCCTGGAAAGAAGATCAGCCCAGAA		
T T E Q L L A L M E S L P G K K I S P E		
1030	1050	1070
GAGATTGAGAGAACGAGAAAGACCTGCAAATCGAGCGAGCAGCTCCTGAAGCTACTCAGT		
E I E R T R K T C K S S E Q L L K L L S		
1090	1110	1130
TTATGGAGGATCAAAATGGTACCAAGACACCTTGAAGGGCCTGATGTATGCCCTCAAG		
L W R I K N G D Q D T L K G L M Y A L K		
1150	1170	1190
CACTTGAAAACATCCCACTTTCACAAACTGTCAACCCACAGTCTGAGGAAGACCATGAGG		
H L K T S H F P K T V T H S L R K T M R		
1210	1230	1250
TTCCCTGCACAGCTTCACAATGTACAGACTGTATCAGAAGCTTTAGAAATGATAGGG		
F L H S F T M Y R L Y Q K L F L E M I G		
1270	1290	1310
AATCAGGTTCAATCCGTGAAAATAAGCTGCTTATAACTAGGAATGGTCACTGGCTGTTT		
N Q V Q S V K I S C L		
CTTCA		

FIG. 9C

10 GTATATATAACGTGATGAGCGTACGGGTGCGGAGACGCACCGGAGCGCTCGCCCAGCCGC  
 30 70 CGYCTCCAAGCCCCCTGAGGTTCCGGGGACCACAATGAACAAGTTGCTGTGCTGCGCGCT  
 50 90 M N K L L C C A L  
 130 150 170 CGTGTTCTGGACATCTCATTAAAGTGGACCACCCAGGAAACGTTCCCTCCAAGTACCT  
 V F L D I S I K W T T O E T F P P K Y L  
 190 210 230 TCATTATGACGAAGAACCTCTCATCAGCTGTTGTGACAAATGTCCCTGGTACCTA  
 H Y D E E T S H Q L L C D K C P P G T Y  
 250 270 290 CCTAAAACAAACACTGTACAGCAAAGTGGAAAGACCGTGTGCGCCCTTGCCCTGACCACTA  
 L K Q H C T A K W K T V C A P C P D H Y  
 310 330 350 CTACACAGACAGCTGGCACACCAGTGACGAGTGTCTATACTGCAGCCCCGTGTGCAAGGA  
 Y T D S W H T S D E C L Y C S P V C K E  
 370 390 410 GCTGCAGTACGTCAAGCAGGAGTGCATCGCACCCACAACCGCGTGTGCGAATGCAAGGA  
 L Q Y V K Q E C N R T H N R V C E C K E  
 430 450 470 AGGGCGCTACCTTGAGATAGAGTTCTGCTTGAAACATAGGAGCTGCCCTGGATTG  
 G R Y L E I E F C L K H R S C P P G F G  
 490 510 530 490 510 530  
 A GTGGTGCAAGCTGGAACCCAGAGCGAAATACAGTTGCAAAAGATGTCCAGATGGTT  
 V V Q A G T P E R N T V C K R C P D G F  
 550 570 590 CTTCTCAAATGAGACGTACATCTAAAGCACCCCTGTAGAAAACACACAAATTGCAGTGTCTT  
 F S N E T S S K A P C R K H T N C S V F  
 610 630 650 TGGTCTCCTGCTAACTCAGAAAGGAAATGCAACACACGACAACATATGTTCCGGAAACAG  
 G L L L T Q K G N A T H D N I C S G N S  
 670 690 710 TGAATCAACTCAAAATGTGGAATAGATGTTACCTGTGAGGAGGCATTCTTCAGGTT  
 E S T Q K C G I D V T L C E E A F F R F  
 730 750 770 TGCTGTTCTACAAAGTTACGCCCTAACTGGCTTAGTGTCTGGTAGACAATTGCCTGG  
 A V P T K F T P N W L S V L V D N L P G

# FIG. 9D

790	810	830
CACCAAAGTAAACGCAGAGAGTGTAGAGAGGATAAAACGGCAACACAGCTCACAAGAACAA		
T K V N A E S V E R I K R Q H S S S Q E Q		
850	870	890
GACTTTCCAGCTGCTGAAGTTATGGAAACATCAAAACAAAGACCAAGATATAGTCAAGAA		
T F Q L L K L W K H Q N K D Q D I V K K		
910	930	950
GATCATCCAAGATATTGACCTCTGTGAAAACAGCGTGAGCGGCACATTGGACATGCTAA		
I I Q D I D L C E N S V Q R H I G H A N		
970	990	1010
CCTCACCTTCGAGCAGCTTCGTAGCTTAGGAAAGCTTACCGGGAAAGAAAGTGGGAGC		
L T F E Q L R S L M E S L P G K K V G A		
1030	1050	1070
AGAAGACATTGAAAAAACAAATAAAGGCATGCAAACCCAGTGACCAGATCCTGAAGCTGCT		
E D I E K T I K A C K P S D Q I L K L L		
1090	1110	1130
CAGTTTGTGGCGAATAAAAATGGCGACCAAGACACCTTGAAGGGCTTAATGCACGCACT		
S L W R I K N G D Q D T L K G L M H A L		
1150	1170	1190
AAAGCACTCAAAGACGTACCACTTTCCCCAAACTGTCACTCAGAGTCTAAAGAACCAT		
K H S K T Y H F P K T V T Q S L K K T I		
1210	1230	1250
CAGGTTCCCTCACAGCTTCACAATGTACAAATTGTATCAGAAGTTATTTAGAAATGAT		
R F L H S F T M Y K L Y Q K L F L E M I		
1270	1290	1310
AGGTAAACCAGGTCCAATCAGTAAAAATAAGCTGCTTATAACTGGAAATGGCCATTGAGCT		
G N Q V Q S V K I S C L		
1330	1350	
GTTCCTCACAAATTGGCGAGATCCCATGGATGATAA		

## FIG.9E

muoste0.frg	M N K W L C C A L L V L L D I E N T T O E T L P P K Y L H Y D P E T G H Q L L C D K C A P G T Y L	50
ratoste0.frg	M N K W L C C A L L V F L D I E N T T O E T F P P K Y L H Y D P E T G R Q L L C D K C A P G T Y L	50
huoste0.frg	M N K W L C C A L V F L D I S I K W I T Q E T F P P K Y L H Y D P E T S H Q L L C D K C P P G T Y L	50
muoste0.frg	K Q H C T V R R K T L C V P C P D H S Y T D S W H T S D E C V Y C S P V C K E L Q S V K Q E C N R T	100
ratoste0.frg	K Q H C T V R R K T L C V P C P D Y S Y T D S W H T S D E C V Y C S P V C K E L Q T V K Q E C N R T	100
huoste0.frg	K Q H C T A K W K T V C A P C P D H Y Y T D S W H T S D E C L Y C S P V C K E L Q Y V K Q E C N R T	100
muoste0.frg	H N R V C E C E E G R Y L E I E F C L K H R S C P P G S G V V Q A G T P E R N T V C K X C P D G F F	150
ratoste0.frg	H N R V C E C E E G R Y L E I E F C L K H R S C P P G I G V I Q A G T P E R N T V C K R C P D G F F	150
huoste0.frg	H N R V C E C E C K E G R Y L E I E F C L K H R S C P P G F G V V Q A G T P E R N T V C K R C P D G F F	150
muoste0.frg	S G E T S S K A P C I K H T N C S T F G I L L I O K G N A T E D N V C S G N R E A T Q K C G I D V T	200
ratoste0.frg	S G E T S S K A P C R A H T N C S S L G I L L I Q K G N A T E D N V C S G N R E A T Q N C G I D V T	200
huoste0.frg	S G E T S S K A P C R K H T N C S S V F G L L I T Q R G N A T E D N I C S G N S E S T Q K C G I D V T	200

## FIG.9F

muosteo.frg	L C E E A F F E R F A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R R H S S Q E Q T	250
ratosteo.frg	L C E E A F F E R F A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R R H S S Q E Q T	250
huosteo.frg	L C E E A F F E R F A V P T K I I P N W L S V L V D N L P G T K V N A E S V E R I K R Q H S S Q E Q T	250
muosteo.frg	F O L L K L W K H Q N R D Q E M V K K I I Q D I D L C E S S V Q R H L G H S N L T E Q I L A L M E	300
ratosteo.frg	F O L L K L W K H Q N R D Q E M V K K I I Q D I D L C E S S V Q R H I G H A N L T E Q I L R I I L M E	300
huosteo.frg	F O L L K L W K H Q N K D Q D T I V K K I I Q D I D L C E N S V Q R H I G H A N L T F E Q I R S S L M E	300
muosteo.frg	S L P G K K I S P E E I E R T R K T C K S S E Q L L K L L S L W R I K N G D Q D T L K G L M Y A L K	350
ratosteo.frg	S L P G K K I S P D E I E R T R K T C K P S E Q L L K L L S L W R I K N G D Q D T L K G L M Y A L K	350
huosteo.frg	S L P G K K V G A E D I E R T T K A C K P S D Q I L K L L S L W R I K N G D Q D T L K G L M H A L K	350
muosteo.frg	H I L K T S H F P K I V T H S L R K T M R F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
ratosteo.frg	H I L K A Y H F P K I V T H S L R K T I R F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
huosteo.frg	H I S K T Y H F P K I V T Q S L K K T I R F L H S F T M Y K L Y Q K L F L E M I G N Q V Q S V K I S C	400
muosteo.frg	L	401
ratosteo.frg	L	401
huosteo.frg	L	401

## FIG. 10

ltnrr C P Q - G K Y I H P O N N S I C C T K C H K G T Y L Y N D C P G P G Q D T D C R E C E S G S F T A S 49  
 humoste P P K Y L H Y D E E T S H Q L L C D K C P P G T Y L K Q H C T A K - W K T V C A P C P D H Y Y T D S 49

ltnrr E N H L R H C L S C S - K C R K E M C Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F 98  
 humoste W H T S D E C L Y C S P V C - K E L Q Y V K - Q E C N R T H N R V C E C K K E G R Y L E I - - E - E 93

ltnrr Q C P N C S L C L N G - T V H L S C Q E K Q N T V C T - C H A G F F L R E - - - N E C V S C 139  
 humoste - C L K H R S C P P G F G V V Q A G T P E R N T V C K R C P D G F F S N E T S S K A P C R K H 139

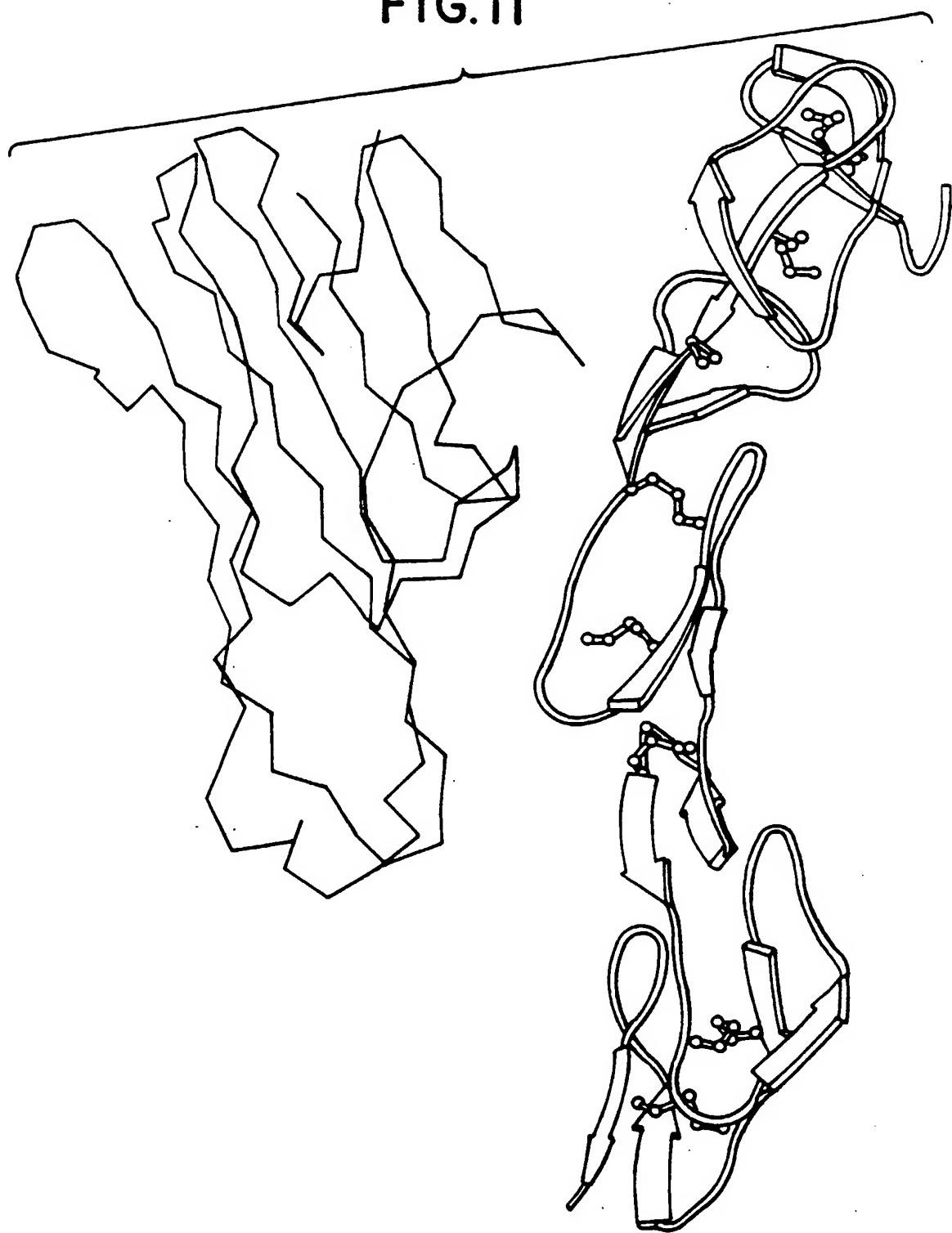
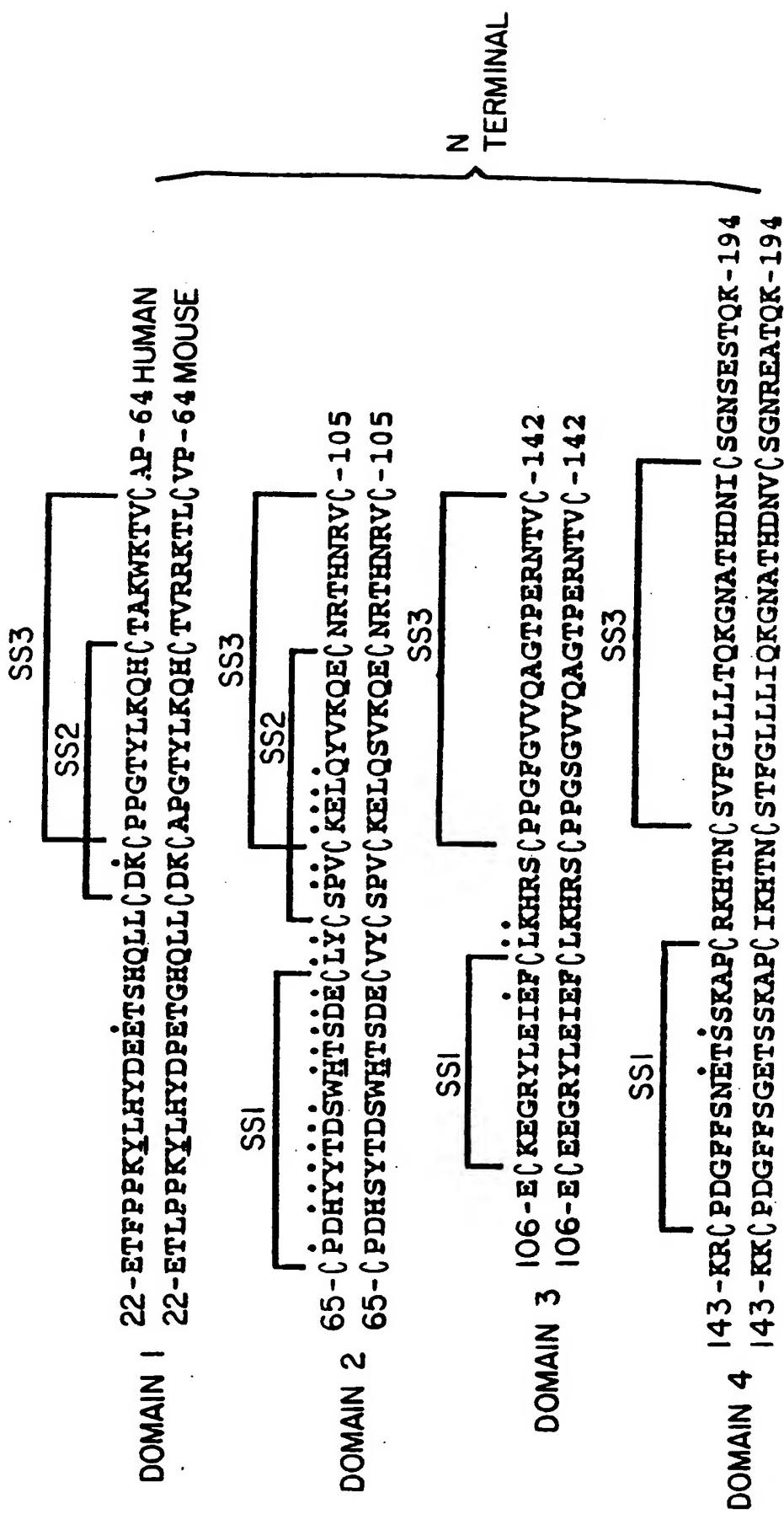
**FIG. II**

FIG. 12A



## FIG. 12B

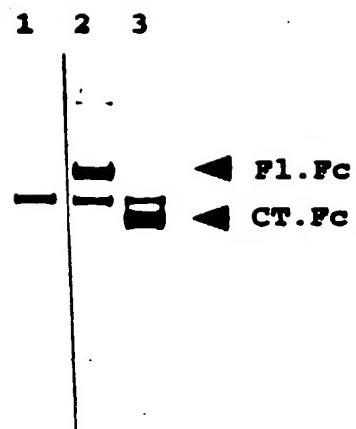
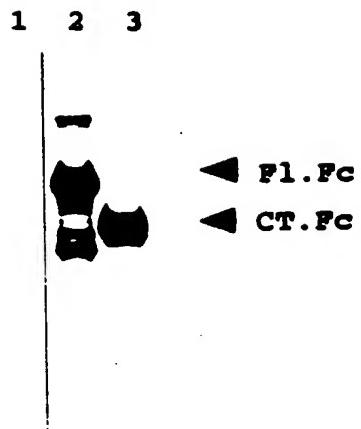
195 - C G I D V T L C E E A F F R A V P T K F T P N W L S V L V D N L P G T K V N A E S V E R I K R Q H S S - 2 4 6  
195 - C G I D V T L C E E A F F R A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R K H S S - 2 4 6

247 - Q E Q T P Q L L K L W I T H Q M K D Q D I V K K I I Q D I D I C E N S V Q R H I G H A N L T P E Q L R S L - 2 9 8  
247 - Q E Q T P Q L L K L W K H Q M R D Q E M V K K I I Q D I D I C E S S V Q R H L G H S N L T T E Q L L A L - 2 9 8

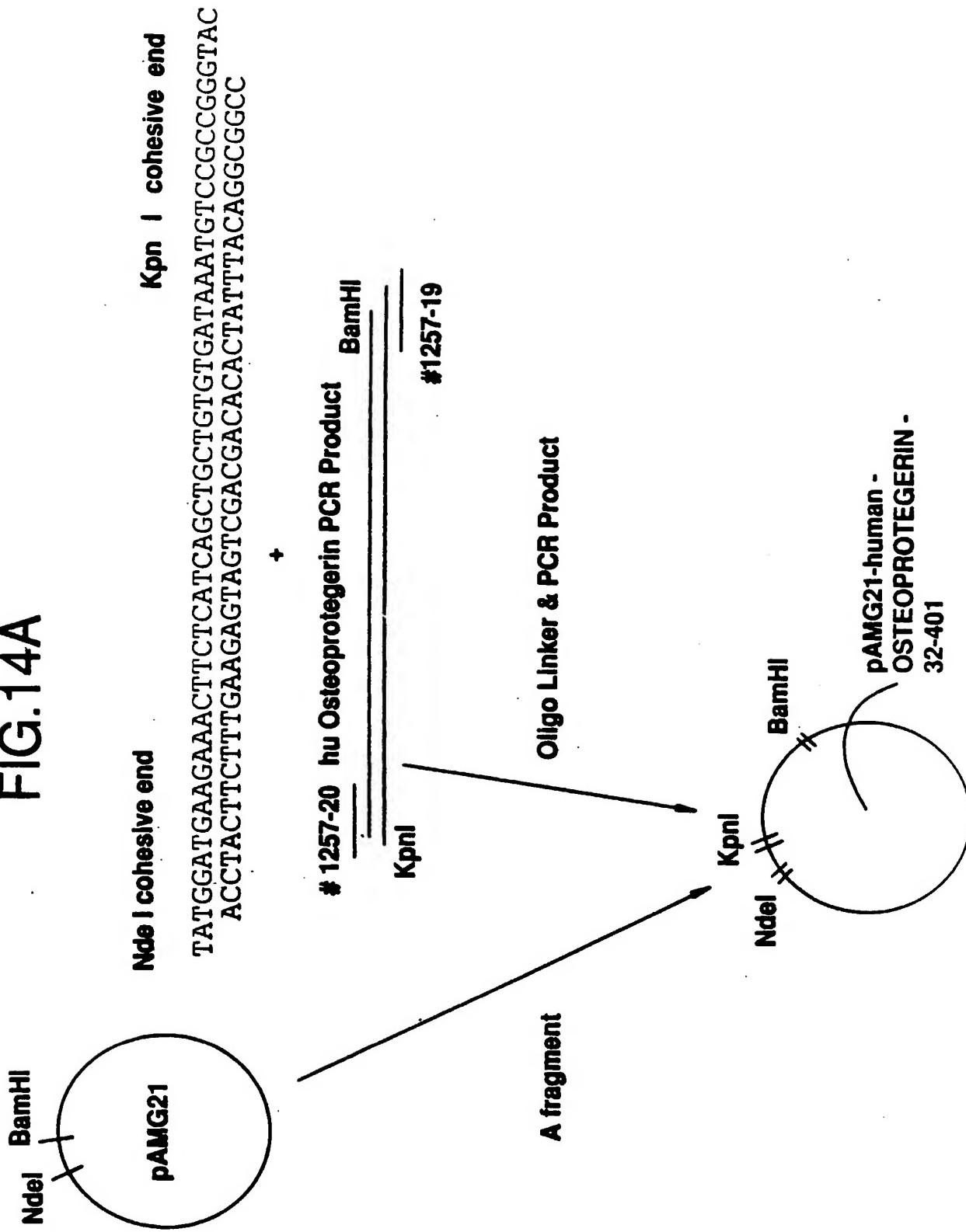
C TERMINAL

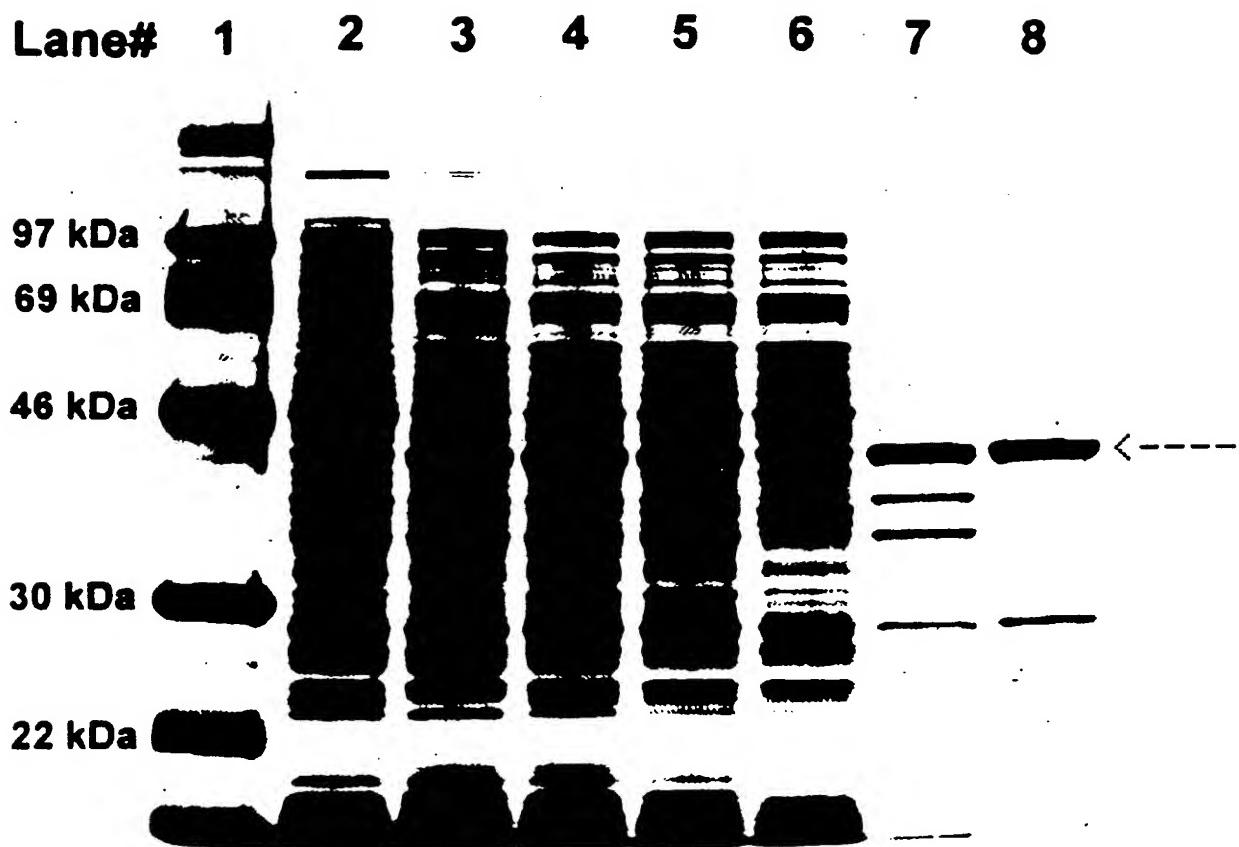
299 - M E S L P G K K V G A E D I E K T I K A C K P S D Q I L K L L S L W R I K N G D Q D T L K G L M H A L K - 3 5 0  
299 - M E S L P G K K I S P E E T E R T R K T C K S S E Q L L K L L S L W R I K N G D Q D T L K G L M Y A L K - 3 5 0

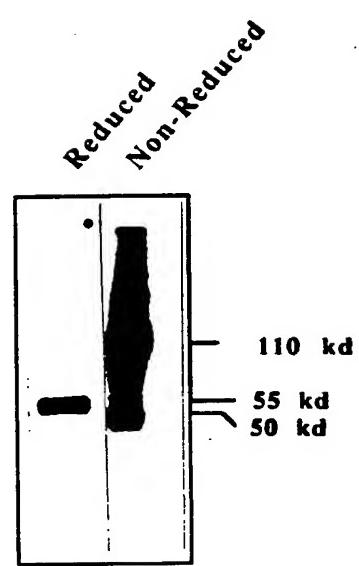
351 - H S K T Y H P P K T V T O S L K K T I R P L H S P T M X K L Y Q K L F L E M I G N Q V Q S V K I S C L - 4 0 1  
351 - H L K T S H P P K T V T H S L R K T M R P L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C L - 4 0 1

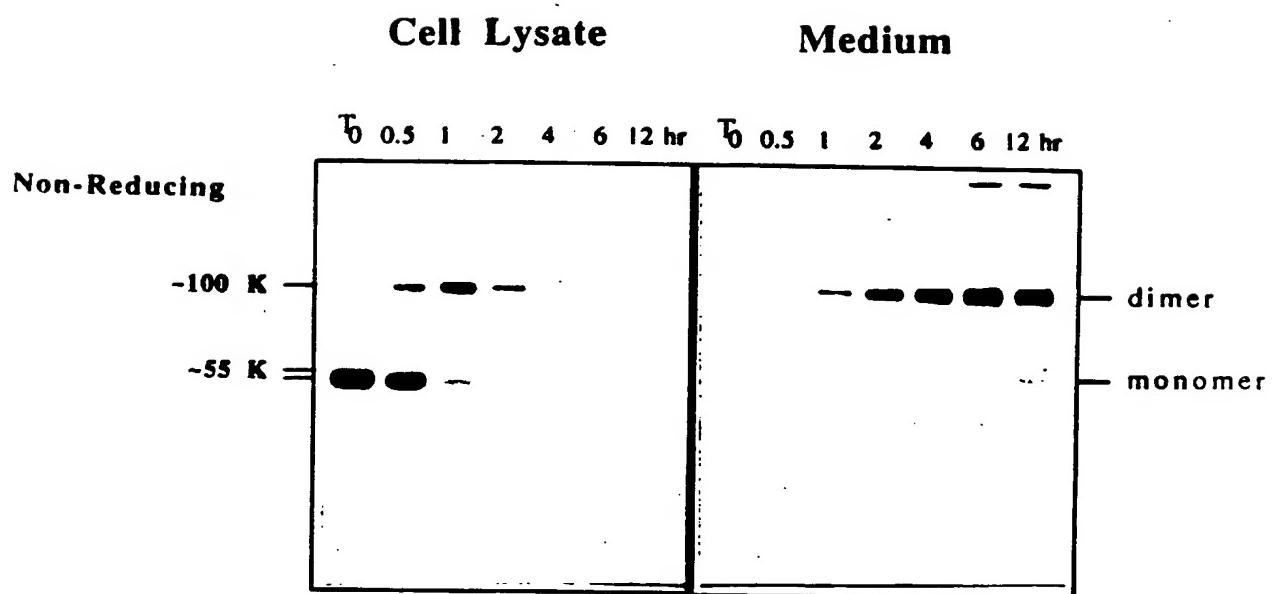
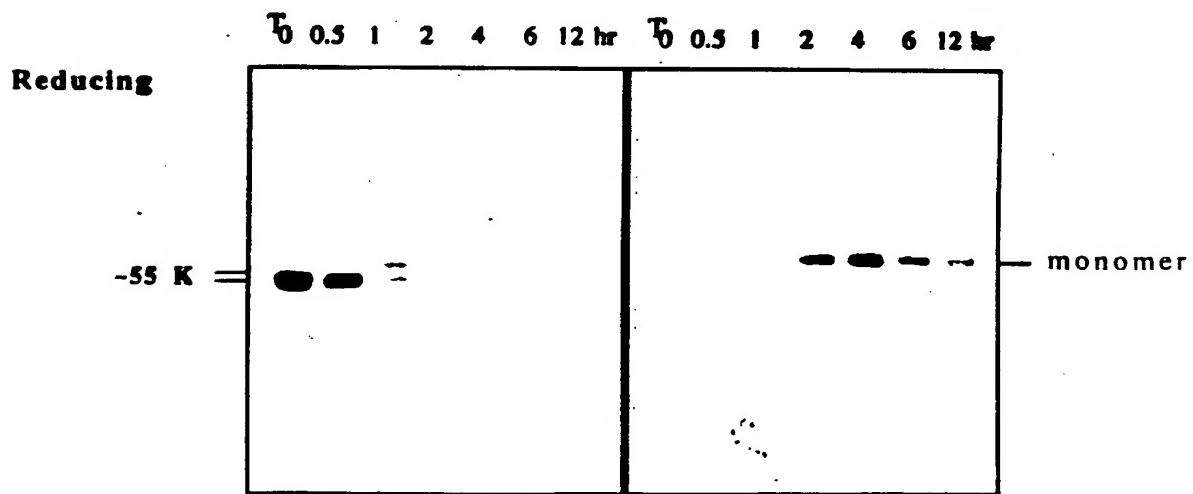
**FIG. 13A****FIG. 13B****FIG. 13C**

# FIG. 14A



**FIG. 14B**

**FIG. 15**

**FIG. 16A****FIG. 16B**

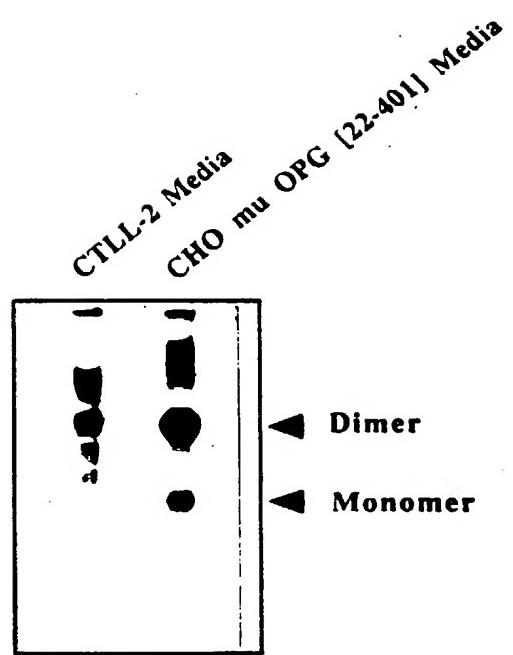
**FIG. 17**

FIG. 18

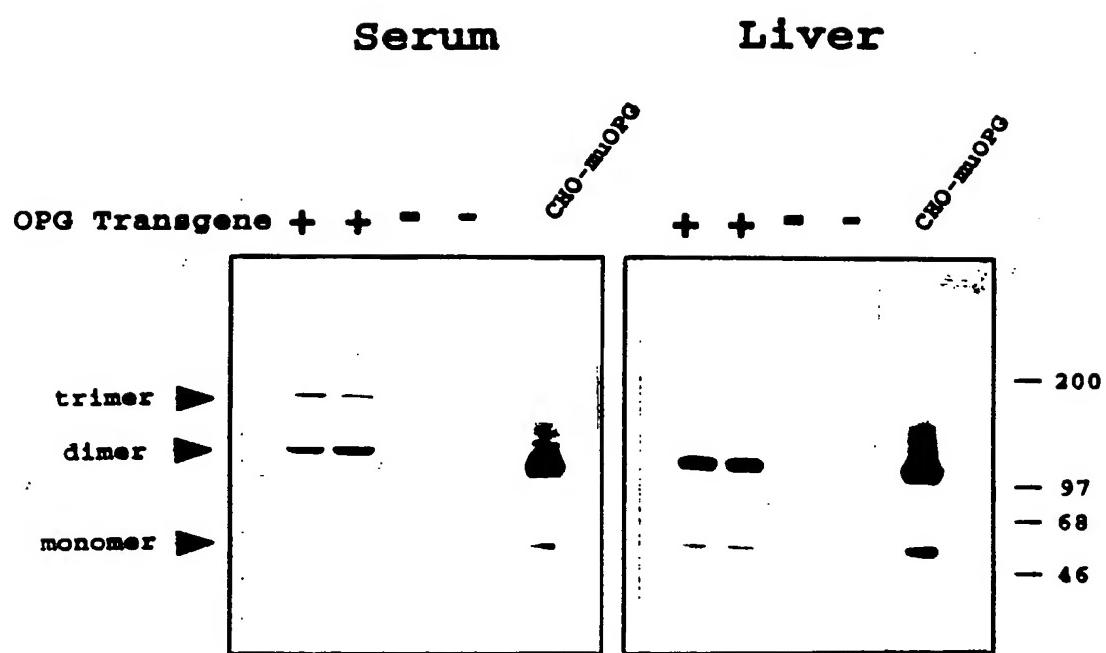
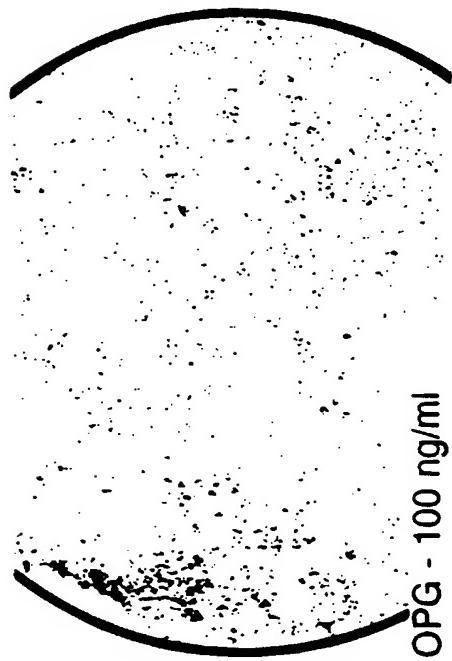


FIG. 19A



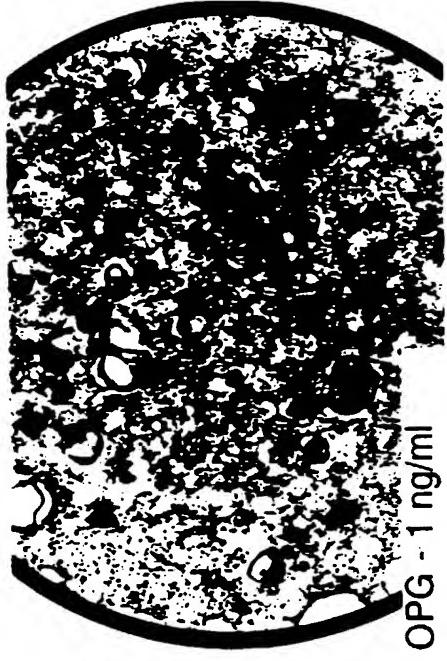
OPG - 100 ng/ml

FIG. 19B



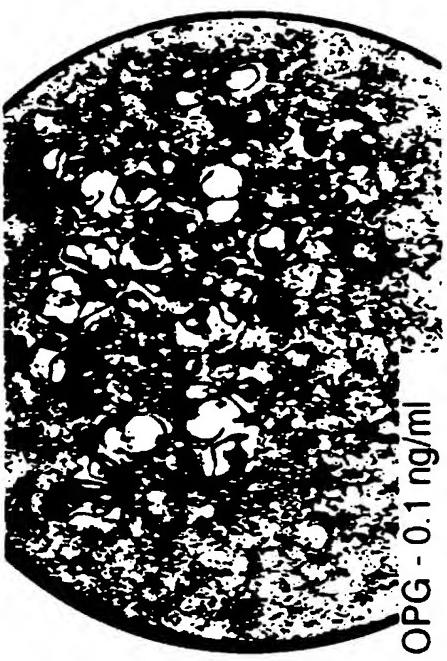
OPG - 10 ng/ml

FIG. 19C



OPG - 1 ng/ml

FIG. 19D



OPG - 0.1 ng/ml

FIG. 19E

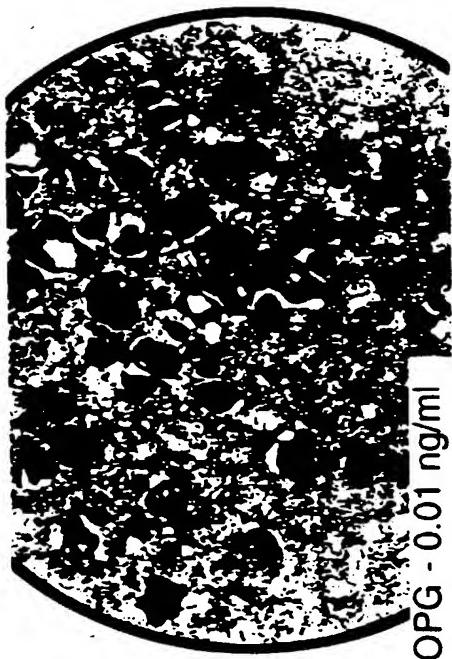
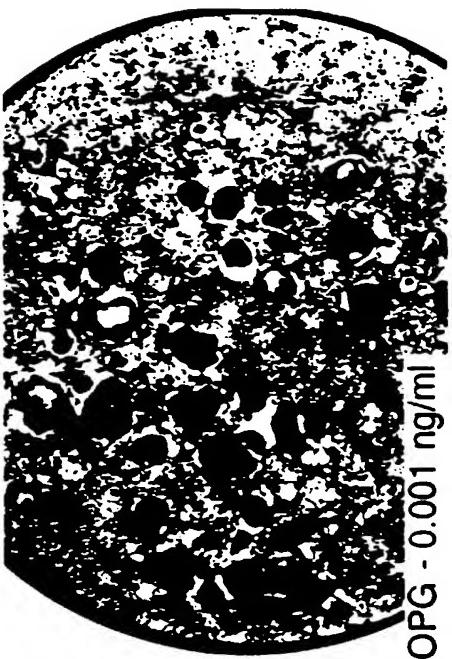


FIG. 19F



Control



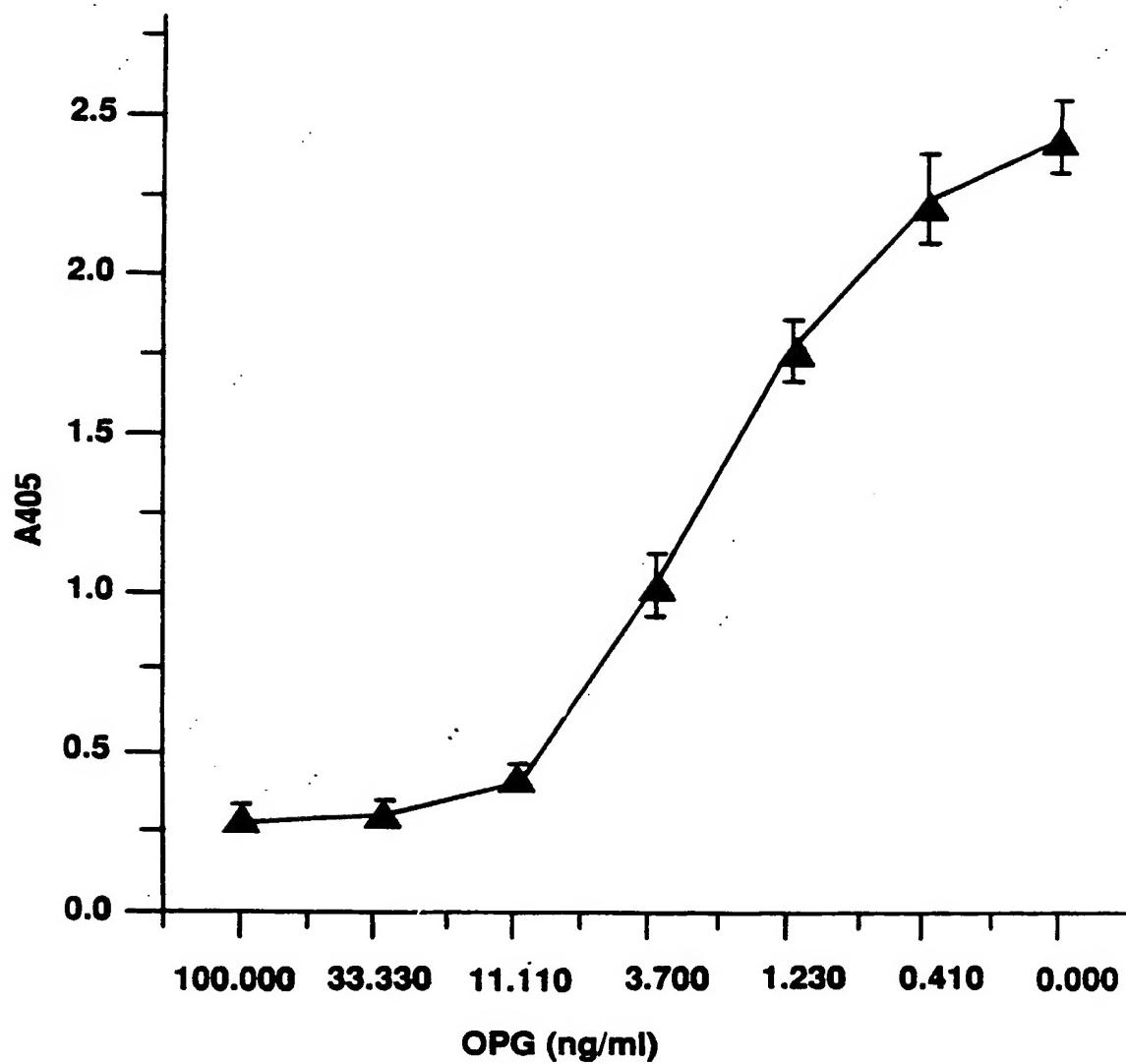
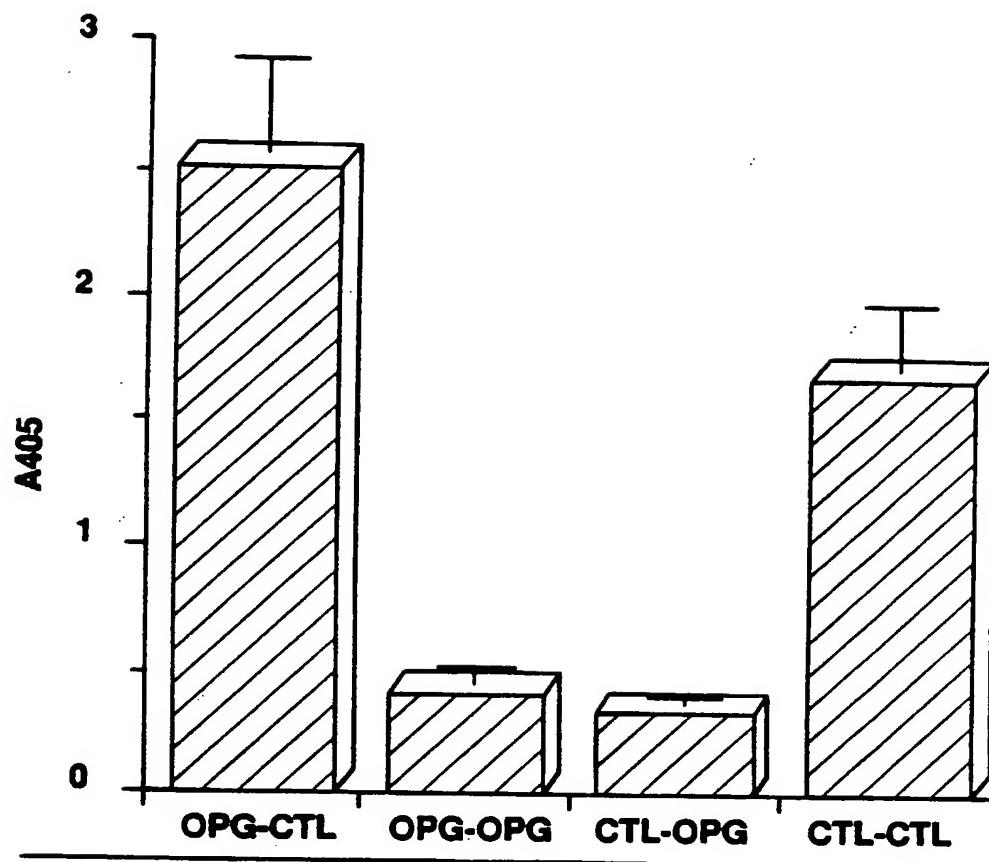
**FIG.20**

FIG.21

**Legend**

**Growth**  
**Bone marrow**  
**cells**  
**CSF -1**

**Intermediate**  
**PGE2 + CSF-1**

**Terminal**  
**ST2 cells**  
**1,25 (OH)<sub>2</sub> D<sub>3</sub>**  
**Dexamethasone**

**4 days****2 days****8 - 10 days****Groups**

CTL - CTL

**OPG****OPG**

OPG - CTL

100 ng/ml

— — —

OPG - OPG

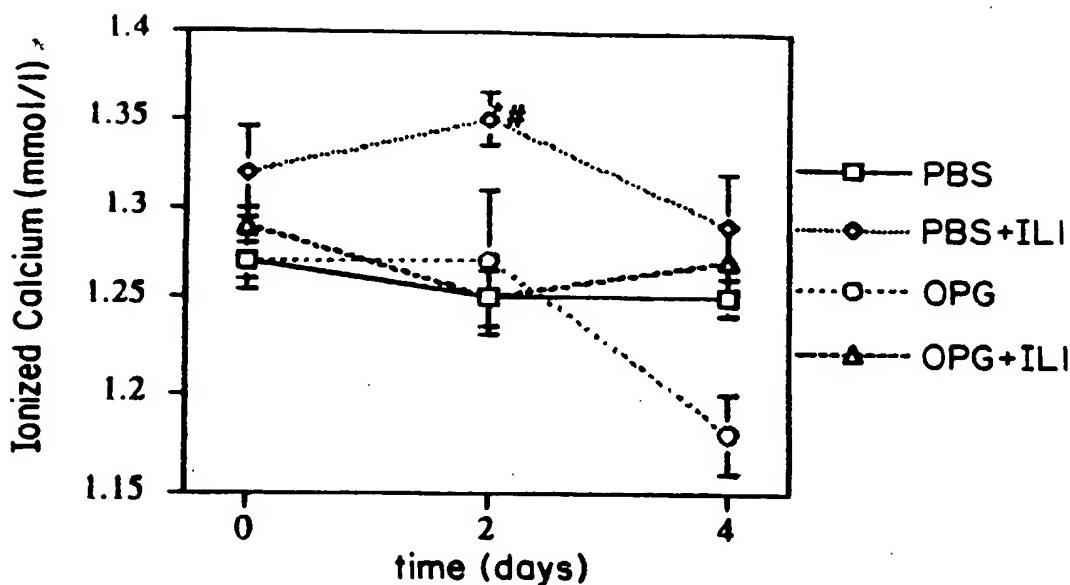
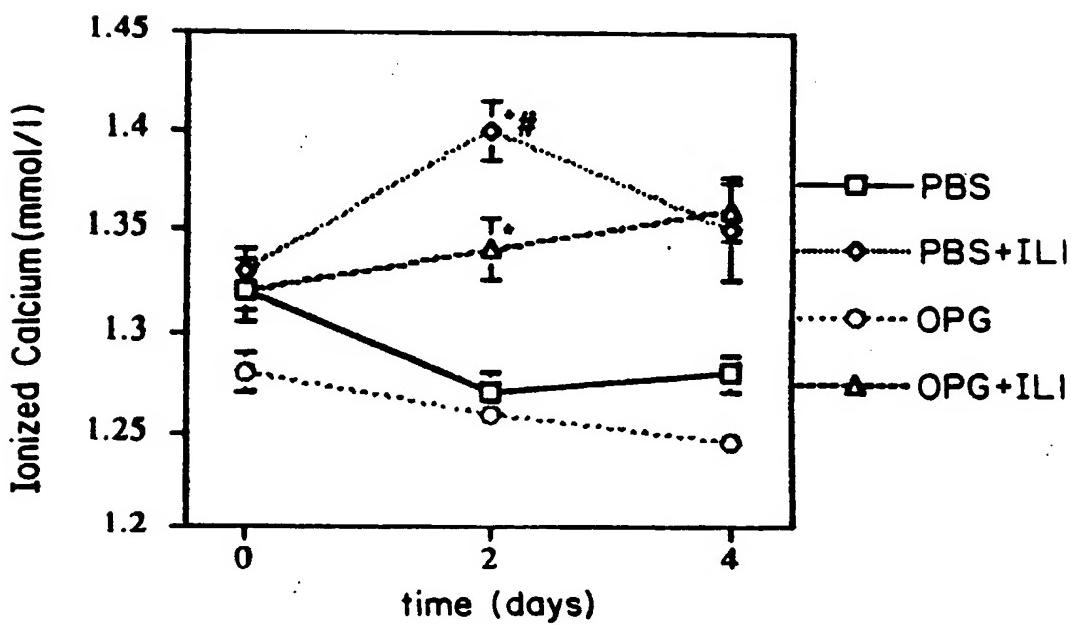
— — —

100 ng/ml

OPG - OPG

100 ng/ml

100 ng/ml

**FIG.22A****FIG.22B**\* Different to PBS,  $p < 0.05$ # Different to OPG + IL1,  $p < 0.05$

**FIG. 23A**

PBS/PBS



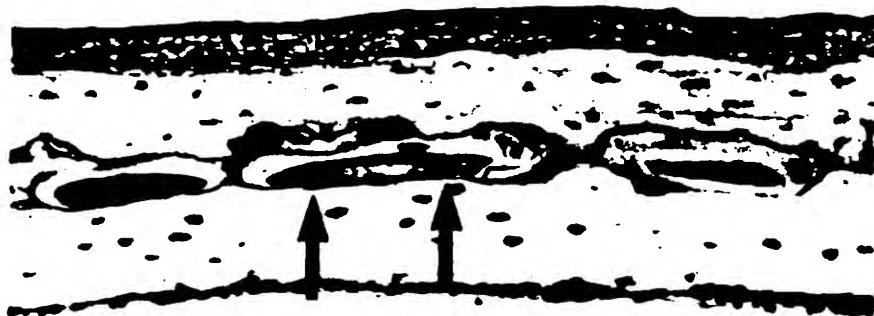
**FIG. 23B**

IL1/PBS



# FIG.23C

PBS/OPG



# FIG.23D

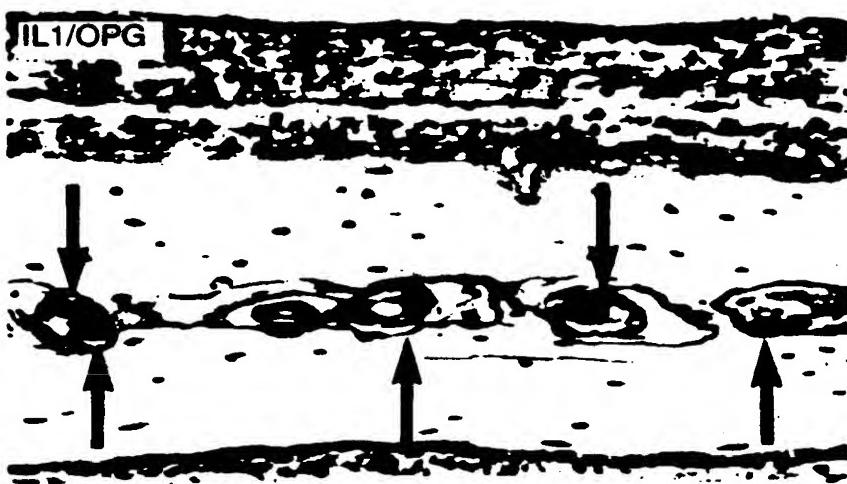


FIG. 24B

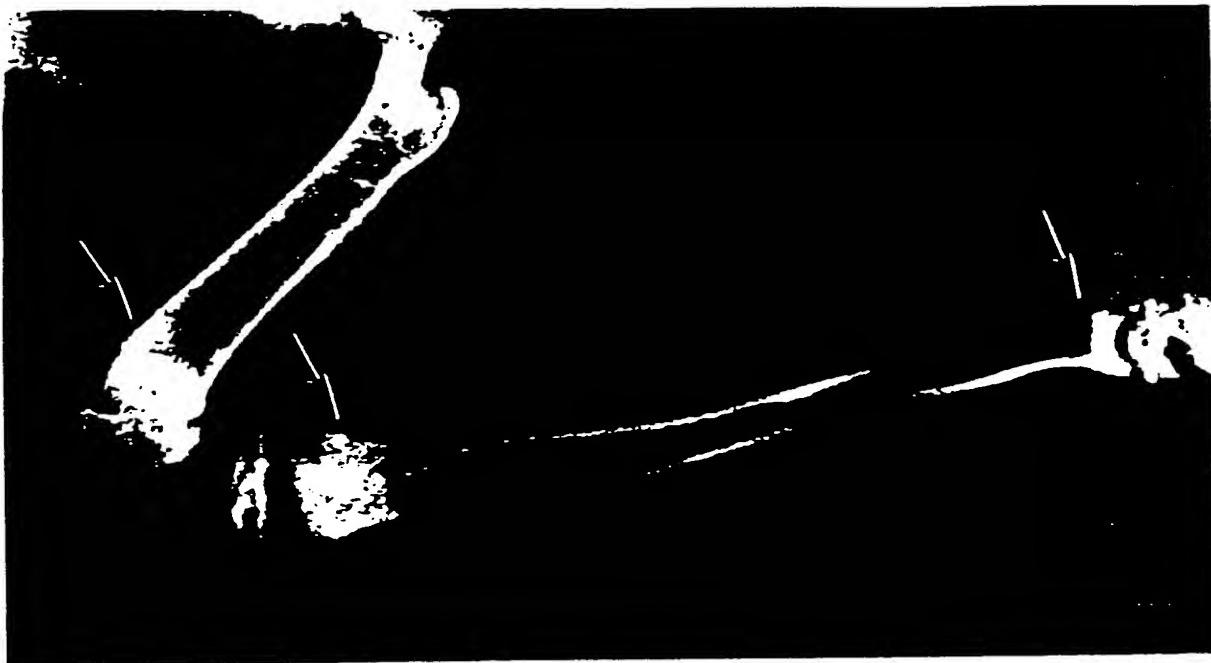
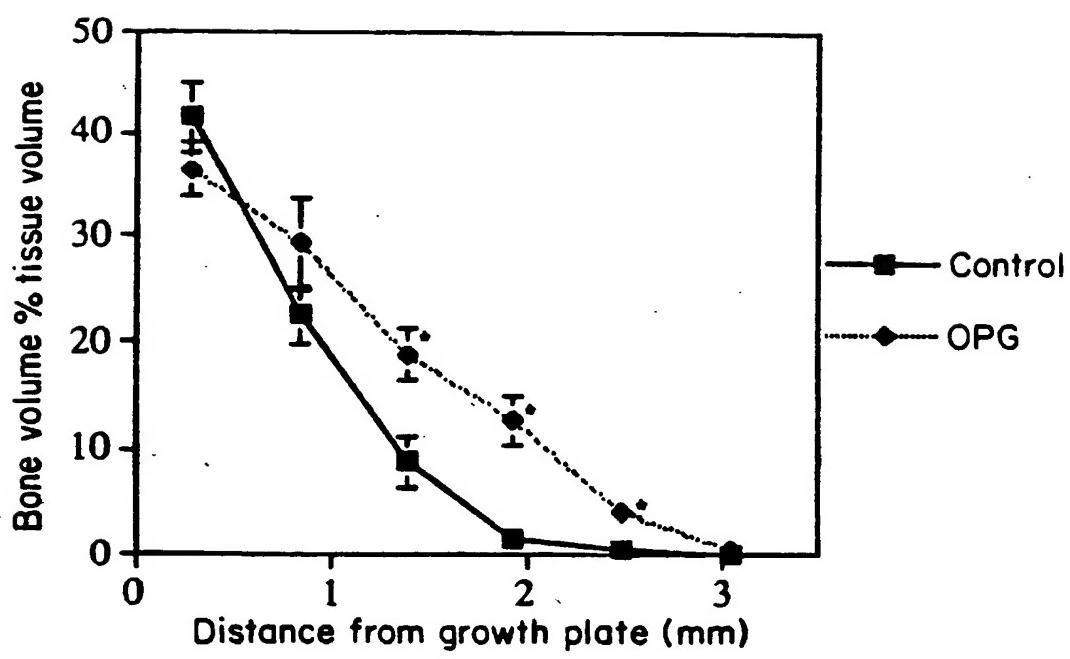


FIG. 24A



**FIG. 25**

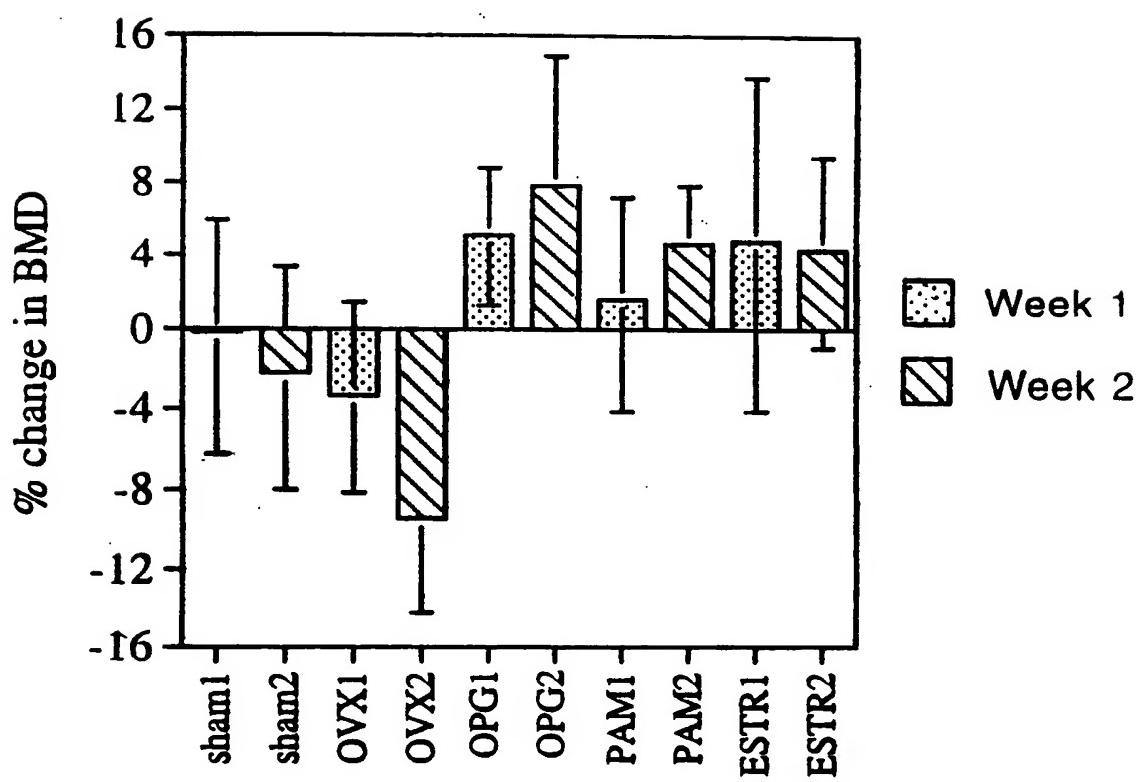
• Different to control  $p < 0.01$

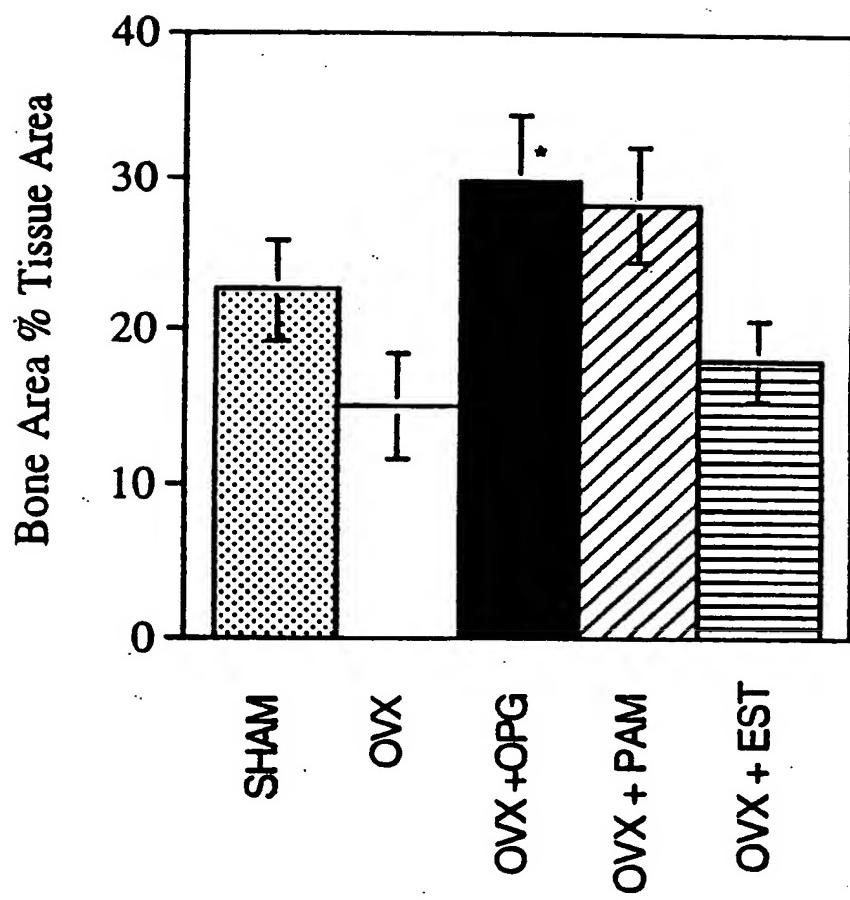
**FIG.26A**



**FIG.26.B**



**FIG. 27**

**FIG.28**

\* Different to OVX  $p < 0.05$

Figure 29A

## DraIII

1 CATGGAAATGTCAGAGTGGAGAACCAACCCAGTGCCACTGCAGCAGTTATTATCA  
 1 GTACCCCTTACAGTCTACCTCTGGTGTGGTCACGGTGACGTGTAACAATAATAGT 60

61 CAAATCCTAATAGTTGCAGTGGGCTTGCTGATGATGGCTACTTGCTCAAAAGAAAA  
 61 GTTTAGGATTATCAAACGTCACCCGAAACGACTACTACCGACTGAACGAGTTTCTTT 120

121 TTAATTGTCAGTGTCTATGGCTTGTGAGATAAAACCCCTTCTTGCCATACCA  
 121 AATTAACAGGTACAGATACCGAAACACTCTATTGGGAGAAAAGAACGGTATGGT 180

181 TTTTAACTGCTTGAGAATATACTGCAGCTTATTGCTTCTCTTATCCTACAATA  
 181 AAAAATTGGACGAAACTCTTATATGACGTCGAAATAACGAAAAGAGGAATAGGATGTTAT 240

241 TAATCAGTAGCTTGATCTTTCATTGAAATGAAATATGGCATTAGCATGACCATAAA  
 241 ATTAGTCATCAGAACTAGAAAAGTAAACCTTACTTTATACCGTAAATCGTACTGGTATTT 300

301 AAGCTGATTCACGGAAATAAAAGCTTTAAATCATCACTCTATCACTGAATTCTAATT  
 301 TTGCACTAAAGGTGACCTTATTTCAGAAAATTAGTAGTAGTGAGATAGTGAATTAGATTAA 360

361 TTTCTGAAAAGTTCAAGCCAGTTACTTTGATAGGATAACGGAAAGGGAGTGAGCCAG  
 361 AAAAGACTTTCAAAGTTCGGTCAATGAAAACATCTAATTGCCCTCCCTCACTCGTC 420

## XcmI

421 TGGGTGAGGTGGTTCCCATGTAGTCATGGCTAATACTGGAGAATCTTATTCTAACCA  
 421 ACCCACTCCACCCAAAGGGTACATCAGTTACCGGATTATGACCTCTTAGAATAAGATTGGT 480

481 AGCCTTCAGAGCAAGCTGTGAGCCCCCTCAGACAGTGGCTACTCATGAGACAGTCATT  
 481 TCGGAAGGTCTCGTCTGACACTCGGGAGTCGTCAACCGATGAGTACTCTGTCAAGGTAA 540

541 GGGGTTAAAGGAAGAAAATATAACTTCTATTCTATTGACATTGACATTGTCTTAGATGC  
 541 CCCCATTTCTCTTTATATTGAAAGATAAAAGATAAGTAAACGTAAACAGAAATCTACG 600

601 CCATTTGGGTGAGTTTATAGAAGTACAGCTACATTAAAAAATAGAACTGATAATAGATA  
 601 GGTAAACCCACTCAAAATATCTCATGTCATGTAATTCTTATCTGACTATTATCTAT 660

661 AGGTTTAAAAAAACTTCATTGACCACTGGTCAAGATTCCATTCAAAGTGAACAAAA  
 661 TCCGAAATTGAAAGTAAAGTAGTGGTCAACAGTTCTAAGGTAAGTTCACTTTT 720

721 CCAATTCTAACGGTTGGTAAACACAGCAGATGGCAGGGGAAAAATTAAAGTGAGTGC  
 721 GGTTAAAGATTGCCAACATTGTGTCTACCGTCCCCTTTAATTCACTCAG 780

781 ATGTACCTTAAAGAAACACTGAAATGCACACACATTACTAACCTGCTCATTCTATTAT  
 781 TACATGAAATTCTTGTGACTTACGTGTGTAAATGAATTGGGACGACTAAAGTAAATA 840

841 TTACATATAGCTGGGTGTACAAAATTAGAAATAATACATATGGGGCGGGGCTTA  
 841 AATGTATATCAGAACCCACATGTTAAATCTTATTATGTATACCCCCGCCCCGGAAT 900

901 GCTGCACAAATAGGATGCGCGGGGGCTTGGTAGGGCGGAGCCTAGCTGCACAAATA  
 901 CGACGTGTTATCCTACGGCGCCGGGAACCATCCCCGCGTCAACGTCAGTGT 960

961 GGATGGCGGGGGGGCTTGGTGGGGGGGGCTAACGCTGCCAAGTGGTACACAGCTCA  
 961 CCTACGGCGCCGGGAACCAACCCCCGCCCCGGATTGACGCGTTCACCATGTGTCAGT 1020

1021 GGGCTGGATTTCGGCCTAAACTTGACGGCAATCTAGCGTGAAGGCTGGTAGGATTAA  
 1021 CCCGACGCTAAAGCGCGTTGAACTGCGTTAGGATGCCACTTCCGACCATCCTAAAAT 1080

Figure 29B

Figure 29C

**Figure 29D**

Figure 29E

Figure 29F

Figure 29G

b K N Q V S L T C L V K G F Y P S D I A V -  
 5831 GGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCCTCCCGTGTGGA + 5880  
 CCTCACCCCTCTCGTTACCGTCGCCCTTGTGATGTTCTGGTGCAGGGCACGACCT  
 b E W E S N G Q P E N N Y K T T P P V L D .

AarI

5881 CTCCGACGGCTCCTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCA + 5940  
 GAGGCTGCCAGGAAGAAGGAGATGTCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGT  
 b S D G S F F L Y S K L T V D K S R W Q Q .

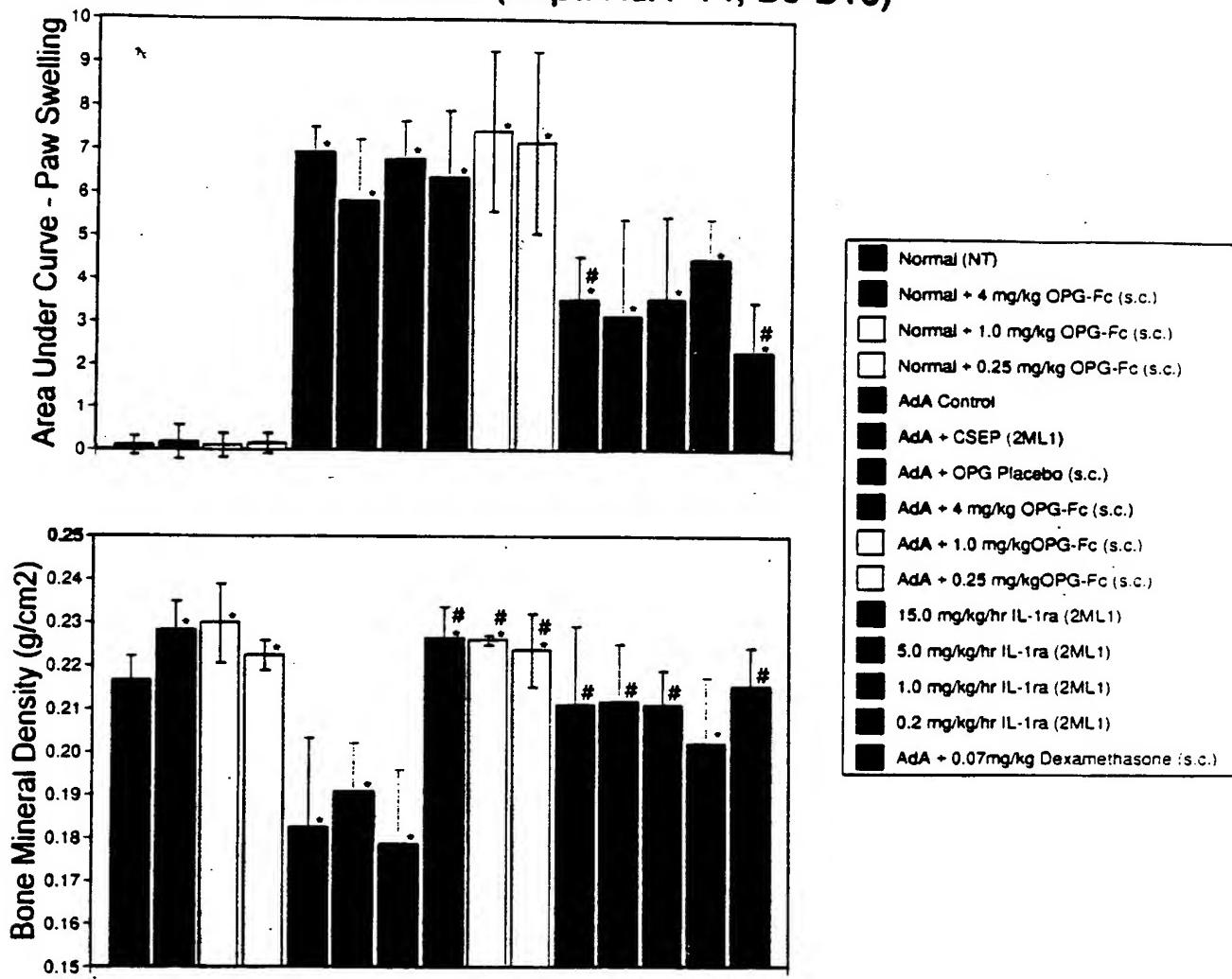
SapI

5941 GGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACACTACACGCAGAA + 6000  
 CCCCTTGCAAGAGATACGAGGCACACTACGTACTCCGAGACGTGTTGGTGTGATGTGGCTTT  
 b G N V F S C S V M H E A L H N H Y T Q K .

6001 GAGCCTCTCCCTGTCTCCGGTAATGATAACTCGAC + 6037  
 CTCGGAGAGGGACAGAGGCCATTACTATTGAGCTG  
 b S L S L S P G K \* \*

Figure 30A

**Effects of OPG-Fc during the course of adjuvant arthritis in male Lewis rats (Expt. AdA- 14, D9-D16)**

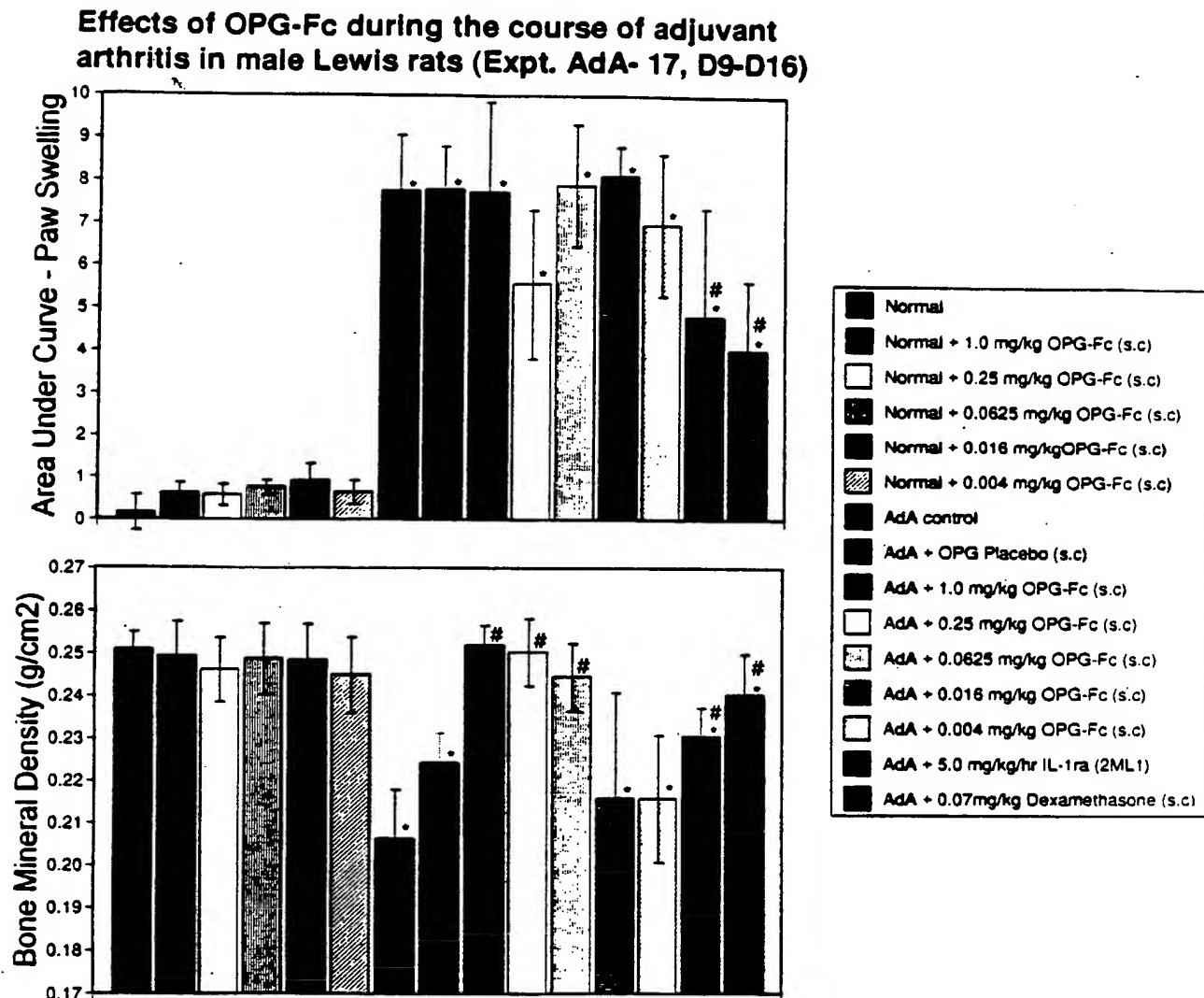


Paws from rats with adjuvant arthritis induced by 0.5mg mycobacteria in oil were analyzed by DEXA for BMD. Evaluation of BMD, a 29mm X 25mm box was centered at the calcaneus (expt AdA-14 2/99, Amgen nb#22957 p47-49).

\* compared to normal, # compared to vehicle

P < 0.05 Mann-Whitney U test

Figure 30B



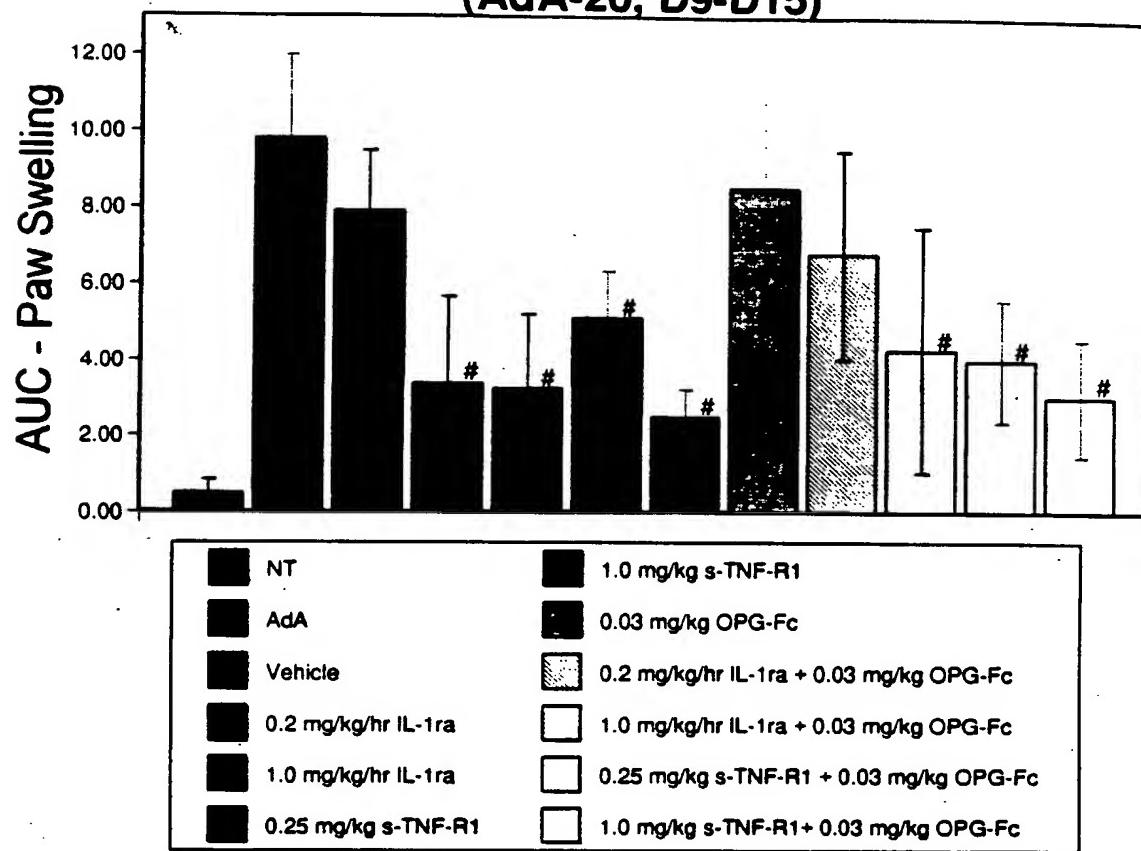
Paws from rats with adjuvant arthritis induced by 0.5mg mycobacteria in oil were analyzed by DEXA for BMD. Evaluation of BMD, a 29mm X 25mm box was centered at the calcaneus (expt AdA -17 3/99, Amgen nb#22957 p62-65).

\* compared to normal, # compared to vehicle

P < 0.05 Mann-Whitney U test

Figure 31A

**Combination treatment with OPG-Fc and IL-1ra or s-TNF-R1 on adjuvant arthritis in male Lewis rats  
(AdA-20, D9-D15)**



Paws from rats with adjuvant arthritis induced by 0.5mg mycobacteria in oil were analyzed by DEXA for BMD. (expt AdA-20 5/99, Amgen nb#22957 p84).

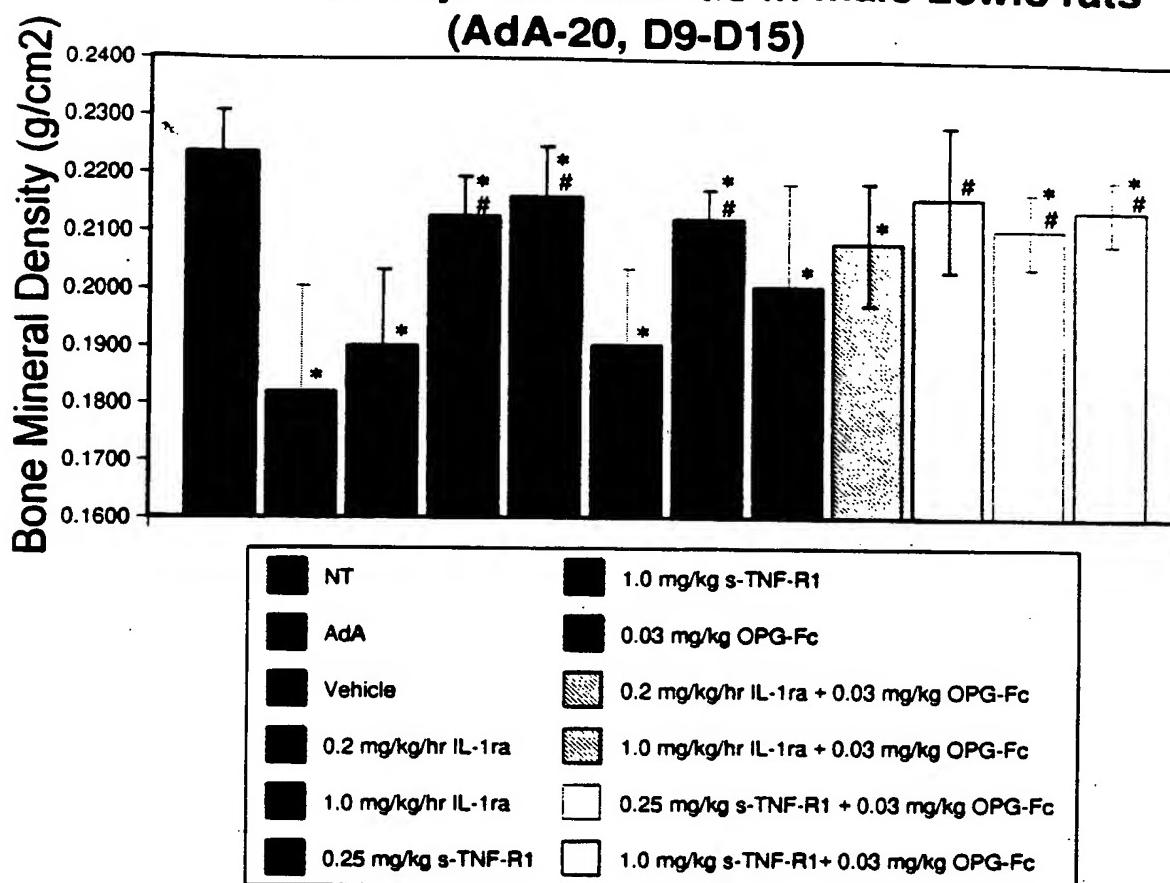
\* compared to normal, # compared to vehicle

P < 0.05 Mann-Whitney U test.

All groups are significant vs normal

Figure 31B

**Combination treatment with OPG-Fc and IL-1ra or s-TNF-R1 on adjuvant arthritis in male Lewis rats  
(AdA-20, D9-D15)**



Paws from rats with adjuvant arthritis induced by 0.5mg mycobacteria in oil were analyzed by DEXA for BMD. Evaluation of BMD, a 29mm X 25mm was centered at the tibiotarsal region. (expt AdA-20 5/99, Amgen nb#22957 p88).

\* compared to normal, # compared to vehicle

.P < 0.05 Mann-Whitney U test.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Amgen Inc.

(ii) TITLE OF INVENTION: OSTEOPROTEGERIN

(iii) NUMBER OF SEQUENCES: 168

## (iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Amgen Inc.
- (B) STREET: 1840 Dehavilland Drive
- (C) CITY: Thousand Oaks
- (D) STATE: California
- (E) COUNTRY: United States
- (F) ZIP: 91320

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Winter, Robert B.
- (C) REFERENCE/DOCKET NUMBER: A-378-CIP2

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAAGGAAGGA AAAAAGCGGC CGCTACANNN NNNNNNT

36

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCCACG CGTCCG

16

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTGCGCAG GC

12

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTAAAACGA CGGCCAGT

18

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGGAAACAG CTATGACC

18

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAATTAACCC TCACTAAAGG

20

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCATTATGAC CCAGAAACCG GAC

23

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGTAGCGCC CTTCCCTCAC A TTC

23

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACTAGTCCC ACAATGAACA AGTGGCTGTG

30

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAAGAATGC GGCGCTAAA CTATGAAACA GCCCAGTGAC CATTC

45

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCTCTAGAA AGAGCTGGGA C 21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCCGTGTTTC CATTATGAG C 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATCAAAGGCA GGGCATACTT CCTG 24

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTGCACTCC TGTTTCACGG TCTG 24

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAAGACACCT TGAAGGGCCT GATG

24

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAACTTTAC AGAACAGCAT CAGC

24

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCGCGGCCG CATGAACAAG TGGCTGTGCT GCG

33

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTCTAGAG AACAGCCCA GTGACCATTC C

31

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTGAAGCTGT GCAAGAACCT GATG

24

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCAAAGGCA GGGCATACTT CCTG

24

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGATCCTGA AGCTGCTCAG TTTG

24

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGCGCGGCCG CGGGGACCAAC AATGAACAAG TTG

33

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGCTCTAGAA TTGTGAGGAA ACAGCTCAAT GGC

33

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATAGCGGCCG CTGAGCCAA ATCTTGAC AAAACTCAC

39

## (2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTAGAGTCG ACTTATCATT TACCCGGAGA CAGGGAGAGG CTCTT

45

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 43 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCTCTGCGGC CGCTAACGAG CTTATTTCA CGGATTGAAC CTG

43

## (2) INFORMATION FOR SEQ ID NO:28:

10

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCCGTAAGAA ACAGCCCCAGT GACC

24

## (2) INFORMATION FOR SEQ ID NO:30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCTCTGCGGC CGCTGTTGCA TTTCCTTCT G

31

## (2) INFORMATION FOR SEQ ID NO:31:

## (i) SEQUENCE CHARACTERISTICS:

11

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His  
1           5           10           15

Gln Leu Leu

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTCTGCGGC CGCACACACG TTGTCATGTG TTGC

34

(2) INFORMATION FOR SEQ ID NO:34:

12

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TCCCTTGCCC TGACCACTCT T

21

## (2) INFORMATION FOR SEQ ID NO:35:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCTCTGCGGC CGCCTTTGC GTGGCTTCTC TGTT

34

## (2) INFORMATION FOR SEQ ID NO:36:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA.

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

## (2) INFORMATION FOR SEQ ID NO:37:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs

13

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCTCTGCGGC CGCTAAGCAG CTTATTTTA CTGAATGG

38

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 37 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCTCTGCGGC CGCCAGGGTA ACATCTATTG CAC

33

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

14

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CCGAAGCTTC CACCATGAAC AAGTGGCTGT GCTGC

35

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCTCTGTCGA CTATTATAAG CAGCTTATTT TCACGGATTG

40

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:  
CCTCTGTCGA CTTAACACAC GTTGTATGT GTTGC

35

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCTCTGTCGA CTTACTTTG CGTGGCTTCT CTGTT

35

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1537 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

16

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTGAAGAGCG GTAAAGAGCGG TTCCCTCCTT CAGCAAAAAA CCCCTCAAGA CCCGTTAGA  
60

GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAAC T CAGCTTCCTT  
120

TCGGGCTTTC TTCTTCTTCT TCTTCTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC  
180

CTGCAGGTCTG ACACTAGTGA GCTCGAATTC CAACGCGTTA ACCATAATGTT ATTCCCTCCTT  
240

TAATTAGTTA AAACAAATCT AGAATCAAAT CGATTAATCG ACTATAACAA ACCATTTCT  
300

TGCGTAAACC TGTACGATCC TACAGGTACT TATGTTAAC AATTGTATTT CAAGCGATAT  
360

AATAGTGTGA CAAAAATCCA ATTTATTAGA ATCAAATGTC AATCTATTAC CGTTTTAATG  
420

ATATATAACA CGCAAAACTT GCGACAAACA ATAGGTAGG ATAAAGAGAT GGGTATGAAA  
480

GACATAAATG CCGACGACAC TTACAGAATA ATTAATAAAA TTAAAGCCTG TAGAAGCAAT  
540

AATGATATTA ATCAATGCTT ATCTGATATG ACTAAAATGG TACATTGTGA ATATTATTTA  
600

CTCGCGATCA TTTATCCTCA TTCTATGGTT AAATCTGATA TTTCAATTCT GGATAATTAC  
660

CCTAAAAAAT GGAGGCAATA TTATGATGAC GCTAATTAA TAAAATATGA TCCTATAGTA  
720

GATTATTCTA ACTCCAATCA TTCACCGATT AATTGGAATA TATTTGAAAA CAATGCTGTA  
780

AATAAAAAT CTCCAAATGT AATTAAGAA GCGAAATCAT CAGGTCTTAT CACTGGTTT  
840

AGTTTCCCTA TTCATACTGC TAATAATGGC TTGGAATGC TTAGTTTGAC ACATTGAGAG  
900

AAAGACAACT ATATAGATAG TTTATTTA CATGCGTGT A TGAAACATACC ATTAATTGTT  
960

CCTTCTCTAG TTGATAATTA TCGAAAATA AATATAGCAA ATAATAATC AAACAAACGAT  
1020

17

TTAACCAAAA GAGAAAAAGA ATGTTTAGCG TGGGCATGCG AAGGAAAAAG CTCTTGGGAT  
1080

ATTTCAAAAA TATTAGGCTG TAGTAAGCGC ACGGTCACTT TCCATTTAAC CAATGCGCAA  
1140

ATGAAACTCA ATACAACAAA CCGCTGCCAA AGTATTCTA AAGCAATTT AACAGGAGCA  
1200

ATTGATTGCC CATACTTTAA AAGTTAAGTA CGACGTCCAT ATTTGAATGT ATTTAGAAAA  
1260

ATAAACAAA GAGTTGTAG AAACGCAAAA AGGCCATCCG TCAGGATGGC CTTCTGCTTA  
1320

ATTTGATGCC TGGCAGTTA TGGCGGGCGT CCTGCCGCC ACCCTCCGGG CCGTTGCTTC  
1380

GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCCCTACTCA GGAGAGCGTT CACCGACAAA  
1440

CAACAGATAA AACGAAAGGC CCAGTCTTC GACTGAGCCT TTCGTTTAT TTGATGCCTG  
1500

GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATAC

1537

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAAATTC GGTAC  
55

## (2) INFORMATION FOR SEQ ID NO:49:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGAATTCCAA CGCGTTAACCC ATATGTTATT CCTCCTTCTA GAATCAAAT

49

## (2) INFORMATION FOR SEQ ID NO:50:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1546 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GCGTAACGTA TGCATGGTCT CCCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA  
60

CGAAAGGCTC AGTCGAAAGA CTGGGCCTTT CGTTTTATCT GTTGTGTC GGTGAACGCT  
120

CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCAGA  
180

GGGTGGCGGG CAGGACGCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGCCATC  
240

CTGACGGATG GCCTTTTGC GTTTCTACAA ACTCTTTGT TTATTTTCT AAATACATTC  
300

AAATATGGAC GTCGTACTTA ACTTTAAAG TATGGGCAAT CAATTGCTCC TGTTAAAATT  
360

GCTTAGAAA TACTTGCGA GCGGTTGTT GTATTGAGTT TCATTGCGC ATTGGTTAAA  
420

TGGAAAGTGA CCGTGCCTT ACTACAGCCT AATATTTTG AAATATCCC AGAGCTTTT  
480

CCTTCGCATG CCCACGCTAA ACATTCTTT TCTCTTTGG TTAAATCGTT GTTGATTAA  
540

TTATTTGCTA TATTATTTT TCGATAATTA TCAACTAGAG AAGGAACAAT TAATGGTATG  
600

TTCATACACG CATGAAAAAA TAAACTATCT ATATAGTTGT CTTCTCTGA ATGTGCAAAA  
660

CTAACGCATTC CGAACCCATT ATTAGCAGTA TGAATAGGGA AACTAAACCC AGTGATAAGA  
720

CCTGATGATT TCGCTTCTTT AATTACATTT GGAGATTTT TATTTACAGC ATTGTTTCA  
780

AATATATTCC AATTAATCGG TGAATGATTG GAGTTAGAAT AATCTACTAT AGGATCATAT  
840

TTTATTAAAT TAGCGTCATC ATAATATTGC CTCCATTTT TAGGGTAATT ATCCAGAATT  
900

GAAATATCAG ATTTAACCAT AGAATGAGGA TAAATGATCG CGAGTAAATA ATATTCACAA  
960

TGTACCATTT TAGTCATATC AGATAAGCAT TGATTAATAT CATTATTGCT TCTACAGGCT  
1020

TTAATTTAT TAATTATTCT GTAAGTGTG TCGGCATTTA TGTCTTCAT ACCCATCTCT  
1080

TTATCCTTAC CTATTGTTG TCGCAAGTTT TGCCTGTTAT ATATCATTAA AACGGTAATA  
1140

GATTGACATT TGATTCTAAT AAATTGGATT TTTGTCACAC TATTATATCG CTTGAAATAC  
1200

AATTGTTAA CATAAGTACC TGTAGGATCG TACAGGTTA CGCAAGAAAA TGGTTGTTA  
1260

TAGTCGATTA ATCGATTTGA TTCTAGATTT GTTTAACTA ATTAAAGGAG GAATAACATA  
1320

20

TGGTTAACGC GTTGGATTG GAGCTCACTA GTGTCGACCT GCAGGGTACC ATGGAAGCTT  
1380

A<sup>C</sup>TCGAGGAT CCCCGGAAAG AAGAAGAAGA AGAAGAAAGC CCGAAAGGAA GCTGAGTTGG  
1440

CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG GCCCTCTAAA CGGGTCTTGA  
1500

GGGGTTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT TCACGC 1546

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 47 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATGAAACAT CATCACCATC ACCATCATGC TAGCGTTAAC GCGTTGG 47

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 49 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AATTCCAACG CGTTAACGCT AGCATGATGG TGATGGTGAT GATGTTCA 49

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 141 base pairs  
(B) TYPE: nucleic acid

21

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CTAATTCCGC TCTCACCTAC CAAACAATGC CCCCTGCAA AAAATAAATT CATATAAAA  
60ACATACAGAT AACCATCTGC GGTGATAAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC  
120

TGGCGGTGAT ACTGAGCACA T

141

(2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 147 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGATGTGCTC AGTATCACCG CCAGTGGTAT TTATGTCAAC ACCGCCAGAG ATAATTTATC  
60ACCGCAGATG GTTATCTGTA TGTTTTTAT ATGAATTTAT TTTTGAGG GGGCATTGT  
120

TTGGTAGGTG AGAGCGGAAT TAGACGT

147

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 55 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

22

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTCCAATTC GGTAC  
53.

## (2) INFORMATION FOR SEQ ID NO:56:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGAATTCCAA CGCGTTAACCC ATATGTTATT CCTCCTTCTA GAATCAAAT

49.

## (2) INFORMATION FOR SEQ ID NO:57:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 668 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GTTGAAGAGCG GAAAGAGCGG TTCCCTCTT CAGCAAAAAA CCCCTCAAGA CCCGTTAGA  
60.

GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAAGT CAGCTTCCTT  
120

TGGGGCTTTC TTCTTCTTCT TCTTCTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC  
180

CTGCAGGTGCG ACACATGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCCTCCTT  
240

TAATTAGTTA ACTCAAATCT AGAATCAAAT CGATAAATTG TGAGCGCTCA CAATTGAGAA  
300

TATTAATCAA GAATTTAGC ATTTGTAAA TGAATTTTT AAAAATTATG AGACGTCCAT  
360

ATTTGAATGT ATTTAGAAAA ATAAACAAAA GAGTTGTAG AAACGCCAAA AGGCCATCCG  
420

TCAGGATGGC CTTCTGCTTA ATTTGATGCC TGGCAGTTA TGGCGGGCGT CCTGCCCGCC  
480

ACCTCCGGG CCGTTGCTTC GCAACGTTCA AATCCGCTCC CGGC GGATTT GTCCTACTCA  
540

GGAGAGCGTT CACCGACAAA CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT  
600

TCGTTTTAT TTGATGCCTG GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATACGTT  
660

ACGCACGT 668

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 726 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GCGTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA  
60

CGAAAGGCTC AGTCGAAAGA CTGGGCCTT CGTTTTATCT GTTGTGGTC GGTGAACGCT  
120

CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTGAACG TTGCGAAGCA ACGGCCCGGA  
180

GGGTGGCGGG CAGGACGCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGGGCCT  
240

CCCACCGCCC GTCCTGCGGG CGGTATTGTA CGGTCCGTAG TTTAATTCTGT CTTCGCCATC  
300

24

CTGACGGATG GCCTTTTGC GTTTCTACAA ACTCTTTGT TTATTTCT AAATACATT  
360

AAATATGGAC GTCTCATAAT TTTTAAAAAA TTCATTTGAC AAATGCTAAA ATTCTTGATT  
420

AATATTCTCA ATTGTGAGCG CTCACAATTT ATCGATTTGA TTCTAGATTT GTTTAACTA  
480

ATTAAAGGAG GAATAACATA TGGTTAACGC GTTCCAATTC GAGCTCACTA GTGTCGACCT  
540

GCAGGGTACC ATGGAAGCTT ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC  
600

CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG  
660

GGCCTCTAAA CGGGTCTTGA GGGTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT  
720

TCACGC

726

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT GGAC

44

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GTCCTCCTGG TACCTACCTA AAACAAC

27

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTACCCGGCG  
60

GACATTTATC ACACAGCAGC TGATGAGAAG TTTCTTCATC CA

102

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Met Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro  
1 5 10 15

Gly Thr Tyr

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid

26

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATGGAAACT TTTCCCTCCAA AATATCTTCA TTATGATGAA GAAACTTCTC ATCAGCTGCT  
60

GTGTGATAAAA TGTCCGCCGG GTAC

84

(2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 78 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CGGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCATAA TGAAGATATT  
60

TTGGAGGAAA AGTTCCA

78

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 44 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TACGCACCTGG ATCCTTATAA GCAGCTTATT TTCACGGATT GAAC

44

(2) INFORMATION FOR SEQ ID NO:66:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

GTGCTCCTGG TACCTACCTA AAACAGCACT GCACAGTG

38

## (2) INFORMATION FOR SEQ ID NO:67:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATGGAAACT CTGCCTCCAA AATACCTGCA TTACGATCCG GAAACTGGTC ATCAGCTGCT  
60

GTGTGATAAA TGTGCTCCGG GTAC

84

## (2) INFORMATION FOR SEQ ID NO:68:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT  
60

TTGGAGGCAG AGTTTCCA

78

## (2) INFORMATION FOR SEQ ID NO:69:

<sup>\*</sup>

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 54 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TATGGACCCA GAAACTGGTC ATCAGCTGCT GTGTGATAAA TGTGCTCCGG GTAC  
54

## (2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 48 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC TGGGTCCA

48

## (2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 87 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

29

TATGAAAGAA ACTCTGCCTC CAAAATACCT GCATTACGAT CCGGAAACTG GTCATCAGCT  
60

GCTGTGTGAT AAATGTGCTC CGGGTAC 87

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 81 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CCGGAGCACCA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT  
60

TTGGAGGCAG AGTTTCTTTC A 81

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GTTCTCCTCA TATGAAACAT CATCACCATC ACCATCATGA AACTCTGCCT CCAAAATACC  
60

TGCATTACGA T 71

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid

30

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

\*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GTTCTCCTCA TATGAAAGAA ACTCTGCCTC CAAAATACCT GCA

43

(2) INFORMATION FOR SEQ ID NO:75:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 76 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TACGCACTGG ATCCTTAATG ATGGTGATGG TGATGATGTA AGCAGCTTAT TTTCACGGAT  
60

TGAACCTGAT TCCCTA

76

(2) INFORMATION FOR SEQ ID NO:76:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 47 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GTTCTCCTCA TATGAAATAC CTGCATTACG ATCCGGAAAC TGGTCAT

47

(2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 43 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

31

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

^

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GTTCTCCTAT TAATGAAATA TCTTCATTAT GATGAAGAAA CTT

43

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

TACGCACGGG ATCCTTATAA GCAGCTTATT TTTACTGATT

40

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

GTTCTCCTCA TATGGAAACT CTGCCTCCAA AATACCTGCA

40

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

32

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TACGGCACTGG ATCCTTATGT TGCATTTCCCT TTCTGAATTAGCA

43

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CCGGAAACAG ATAATGAG

18

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GATCCTCATT ATCTGTTT

18

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCGGAAACAG AGAAGCCACG CAAAAGTAAG

30

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

GATCCTTACT TTTGCGTGGC TTCTCTGTTT

30

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATGTTAACG AG

12

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

34

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GATCCTCATT AACA

14

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

TATGTTCCGG AAACAGTTAA G

21

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

GATCCTTAAC TGTTTCCGGA ACA

23

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

35

TATGTTCCGG AAACAGTGAA TCAACTCAAA AATAAG

36

## (2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GATCCTTATT TTTGAGTTGA TTCACTGTTT CCGGAACA

38

## (2) INFORMATION FOR SEQ ID NO:91:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 100 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

CTAGCGACGA CGACGACAAA GAAACTCTGC CTCCAAAATA CCTGCATTAC GATCCGGAAA  
60

CTGGTCATCA GCTGCTGTGT GATAAATGTG CTCCGGGTAC

100

## (2) INFORMATION FOR SEQ ID NO:92:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 92 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

36

CCGGAGCAC A TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT  
60

TTGGAGGCAG AGTTCTTTG TCGTCGTCGT CG

92

## (2) INFORMATION FOR SEQ ID NO:93:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ACAAACACAA TCGATTTGAT ACTAGA

26

## (2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TTTGTTTTAA CTAATTAAG GAGGAATAAA ATATGAGAGG ATCGCATCAC

50

## (2) INFORMATION FOR SEQ ID NO:95:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

CAT~~A~~ACCATC ACGAAACCTT CCCGCCGAAA TACCTGCACT ACGACGAAGA

50

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

AACCTCCCAC CAGCTGCTGT GCGACAAATG CCCGCCGGGT ACCCAAACA

49

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

TGTTTGGGTA CCCGGCGGGC ATTTGT

26

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CGCACAGCAG CTGGTGGGAG GTTTCTTCGT CGTAGTGCAG GTATTCGGC

50

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GGGAAGGTTT CGTGATGGTG ATGGTGATGC GATCCTCTCA TATTTTATT

49

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

CCTCCTTTAA TTAGTTAAAA CAAATCTAGT ATCAAATCGA TTGTGTTGT

50

(2) INFORMATION FOR SEQ ID NO:101:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

39

ACAAACACAA TCGATTTGAT ACTAGATTG TTTTAACCAA TTAAAGGAGG AATAAAATG  
59

## (2) INFORMATION FOR SEQ ID NO:102:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 48 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

CTAATTAAAG GAGGAATAAA ATGAAAGAAA CTTTCCTCC AAAATATC

48

## (2) INFORMATION FOR SEQ ID NO:103:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

TGTTTGGGTA CCCGGCGGAC ATTTATCACAC

31

## (2) INFORMATION FOR SEQ ID NO:104:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 59 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

40

ACAAACACAA TCGATTTGAT ACTAGATTTG TTTTAACCAA TTAAAGGGAGG AATAAAATG  
59

## (2) INFORMATION FOR SEQ ID NO:105:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

CTAATTAAAG GAGGAATAAA ATGAAAAAAA AAGAAACTTT TCCTCCAAAA TATC  
54

## (2) INFORMATION FOR SEQ ID NO:106:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C

31

## (2) INFORMATION FOR SEQ ID NO:107:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

41

CAGCCCCGGT AAAATGGAAA CGTTTCCTCC AAAATATCTT CATT

44

## (2) INFORMATION FOR SEQ ID NO:108:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

CGTTTCCATT TTACCCGGGC TGAGCGAGAG GCTCTTCTGC GTGT

44

## (2) INFORMATION FOR SEQ ID NO:109:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

CGCTCAGCCC GGGTAAATG GAAACGTTGC CTCCAAAATA CCTGC

45

## (2) INFORMATION FOR SEQ ID NO:110:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

CCATTTTACC CGGGCTGAGC GAGAGGGCTCT TCTGCGTGT

39

42

## (2) INFORMATION FOR SEQ ID NO:111:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

GAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

## (2) INFORMATION FOR SEQ ID NO:112:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

CAGCTGCAGC TAAGCAGCTT ATTTTCACGG ATTG

34

## (2) INFORMATION FOR SEQ ID NO:113:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

AAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

## (2) INFORMATION FOR SEQ ID NO:114:

43

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

CAGCTGCAGC TAAGCAGCTT ATTTTTACTG ATTGG

35

(2) INFORMATION FOR SEQ ID NO:115:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 102 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

CTAGAAAGGAG GAATAACATA TGGAAACTTT TGCTCCAAAA TATCTTCATT ATGATGAAGA  
60

AACTAGTCAT CAGCTGCTGT GTGATAAATG TCCGCCGGGT AC

102

(2) INFORMATION FOR SEQ ID NO:116:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

44

CCGGCGGACA TTTATCACAC AGCAGCTGAT GACTAGTTTC TTCATCATAA TGAAGATATT  
60

TTGGAGCAAA AGTTTCCATA TGTTATTCCT CCTT

94

(2) INFORMATION FOR SEQ ID NO:117:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

CTAGAAGGAG GAATAACATA TGGAAACTTT TCCTGCTAAA TATCTTCATT ATGATGAAGA  
60

AA

62

(2) INFORMATION FOR SEQ ID NO:118:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

CTAGTTTCTT CATCATAATG AAGATATTAA GCAGGAAAAG TTTCCATATG TTATTCCTCC  
60

TT

62

(2) INFORMATION FOR SEQ ID NO:119:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: protein

x

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

Tyr His Tyr Tyr Asp Gln Asn Gly Arg Met Cys Glu Glu Cys His Met  
1 5 10 15

Cys Gln Pro Gly His Phe Leu Val Lys His Cys Lys Gln Pro Lys Arg  
20 25 30

Asp Thr Val Cys His Lys Pro Cys Glu Pro Gly Val Thr Tyr Thr Asp  
35 40 45

Asp Trp His  
50

(2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2432 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 124..1326

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

ATCAAAGGCA GGGCATACTT CCTGTTGCC AGACCTTATA TAAAACGTCA TGTCGCCTG  
60

GGCAGCAGAG AAGCACCTAG CACTGGCCA GCGGCTGCCG CCTGAGGTTT CCAGAGGACC  
120

ACA ATG AAC AAG TGG CTG TGC TGT GCA CTC CTG GTG TTC TTG GAC ATC  
168

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile  
1 5 10 15

ATT GAA TGG ACA ACC CAG GAA ACC TTT CCT CCA AAA TAC TTG CAT TAT  
216

Ile Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr  
20            25            30

GAC CCA GAA ACC GGA CGT CAG CTC TTG TGT GAC AAA TGT GCT CCT GGC  
264

Asp Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly  
35            40            45

ACC TAC CTA AAA CAG CAC TGC ACA GTC AGG AGG AAG ACA CTG TGT GTC  
312

Thr Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val  
50            55            60

CCT TGC CCT GAC TAC TCT TAT ACA GAC AGC TGG CAC ACG AGT GAT GAA  
360

Pro Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu  
65            70            75

TGC GTG TAC TGC AGC CCC GTG TGC AAG GAA CTG CAG ACC GTG AAA CAG  
408

Cys Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln  
80            85            90            95

GAG TGC AAC CGC ACC CAC AAC CGA GTG TGC GAA TGT GAG GAA GGG CGC  
456

Glu Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg  
100            105            110

TAC CTG GAG CTC GAA TTC TGC TTG AAG CAC CGG AGC TGT CCC CCA GGC  
504

Tyr Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly  
115            120            125

TTG GGT GTG CTG CAG GCT GGG ACC CCA GAG CGA AAC ACG GTT TGC AAA  
552

Leu Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys  
130            135            140

AGA TGT CCG GAT GGG TTC TTC TCA GGT GAG ACG TCA TCG AAA GCA CCC  
600

Arg Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro  
145            150            155

TGT AGG AAA CAC ACC AAC TGC AGC TCA CTT GGC CTC CTG CTA ATT CAG  
648

Cys Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln  
160            165            170            175

AAA GGA AAT GCA ACA CAT GAC AAT GTA TGT TCC GGA AAC AGA GAA GCA  
 696  
 Lys Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala  
     180           185           190  
 ACT CAA AAT TGT GGA ATA GAT GTC ACC CTG TGC GAA GAG GCA TTC TTC  
 744  
 Thr Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe  
     195           200           205  
 AGG TTT GCT GTG CCT ACC AAG ATT ATA CCG AAT TGG CTG AGT GTT CTG  
 792  
 Arg Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu  
     210           215           220  
 GTG GAC AGT TTG CCT GGG ACC AAA GTG AAT GCA GAG AGT GTA GAG AGG  
 840  
 Val Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg  
     225           230           235  
 ATA AAA CGG AGA CAC AGC TCG CAA GAG CAA ACT TTC CAG CTA CTT AAG  
 888  
 Ile Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys  
     240           245           250           255  
 CTG TGG AAG CAT CAA AAC AGA GAC CAG GAA ATG GTG AAG AAG ATC ATC  
 936  
 Leu Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile  
     260           265           270  
 CAA GAC ATT GAC CTC TGT GAA AGC AGT GTG CAA CGG CAT ATC GGC CAC  
 984  
 Gln Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His  
     275           280           285  
 GCG AAC CTC ACC ACA GAG CAG CTC CGC ATC TTG ATG GAG AGC TTG CCT  
 1032  
 Ala Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro  
     290           295           300  
 GGG AAG AAG ATC AGC CCA GAC GAG ATT GAG AGA ACG AGA AAG ACC TGC  
 1080  
 Gly Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys  
     305           310           315  
 AAA CCC AGC GAG CAG CTC CTG AAG CTA CTG AGC TTG TGG AGG ATC AAA  
 1128  
 Lys Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys  
     320           325           330           335  
 AAT GGA GAC CAA GAC ACC TTG AAG GGC CTG ATG TAC GCA CTC AAG CAC  
 1176  
 Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His

48

340 345 350

TTG AAA GCA TAC CAC TTT CCC AAA ACC GTC ACC CAC AGT CTG AGG AAG  
1224  
Leu Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys  
355 360 365

ACC ATC AGG TTC TTG CAC AGC TTC ACC ATG TAC CGA TTG TAT CAG AAA  
1272  
Thr Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys  
370 375 380

CTC TTT CTA GAA ATG ATA GGG AAT CAG GTT CAA TCA GTG AAG ATA AGC  
1320  
Leu Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser  
385 390 395

TGC TTA TAGTTAGGAA TGGTCACTGG GCTGTTCTT CAGGATGGC CAACACTGAT  
1376  
Cys Leu  
400

GGAGCAGATG GCTGCTTCTC CGGCTCTGA AATGGCAGTT GATTCCCTTC TCATCAGTTG  
1436

GTTGGAAATGA AGATCCTCCA GCCAACACA CACACTGGGG AGTCTGAGTC AGGAGAGTGA  
1496

GGCAGGCTAT TTGATAATTG TGCAAAGCTG CCAGGTGTAC ACCTAGAAAG TCAAGCACCC  
1556

TGAGAAAGAG GATATTTTA TAACCTCAAA CATAGGCCCT TTCCTTCCTC TCCTTATGGA  
1616

TGAGTACTCA GAAGGCTTCT ACTATCTTCT GTGTCATCCC TAGATGAAGG CCTCTTTAT  
1676

TTATTTTTTT ATTCTTTTT TC GGAGCTGG GGACCGAACCC CAGGGCCTTG CGCTTGCGAG  
1736

GCAAGTGCTC TACCACTGAG CTAAATCTCC AACCCCTGAA GGCTCTTTC TTTCTGCCTC  
1796

TGATAGTCTA TGACATTCTT TTTCTACAA TTGCGTATCAG GTGCACGAGC CTTATCCCAT  
1856

TTGTAGGTTT CTAGGCAAGT TGACCGTTAG CTATTTTCC CTCTGAAGAT TTGATTGAG  
1916

TTGCAGACTT GGCTAGACAA GCAGGGTAG GTTATGGTAG TTTATTTAAC AGACTGCCAC  
1976

CAGGAGTCCA GTGTTCTTG TTCCTCTGTA GTTGTACCTA AGCTGACTCC AACTACATTT  
2036

AGTATGAAAA ATAATCAACA AATTTATTC CTTCTATCAA CATTGGCTAG CTTTGTTC  
2096

GGGCACTAAA AGAAACTACT ATATGGAGAA AGAATTGATA TTGCCCCCAA CGTCAACAA  
2156

CCCAATAGTT TATCCAGCTG TCATGCCCTGG TTCAGTGTCT ACTGACTATG CGCCCTCTTA  
2216

TTACTGCATG CAGTAATTCA ACTGGAAATA GTAATAATAA TAATAGAAAT AAAATCTAGA  
2276

CTCCATTGGA TCTCTCTGAA TATGGAAATA TCTAACTTAA GAAGCTTGA GATTCAGTT  
2336

GTGTTAAAGG CTTTTATTAA AAAGCTGATG CTCTCTGTA AAAGTTACTA ATATATCTGT  
2396

AAGACTATTAA CAGTATTGCT ATTTATATCC ATCCAG 2432

(2) INFORMATION FOR SEQ ID NO:121:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 401 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Met	Asn	Lys	Trp	Leu	Cys	Cys	Ala	Leu	Leu	Val	Phe	Leu	Asp	Ile	Ile
1	5			10											

Glu	Trp	Thr	Thr	Gln	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp
20				25											

Pro	Glu	Thr	Gly	Arg	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	Thr
35				40											

Tyr	Leu	Lys	Gln	His	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro
50				55			60								

Cys	Pro	Asp	Tyr	Ser	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys
65				70			75			80					

50

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu  
85 90 95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr  
100 105 110

Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu  
115 120 125

Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg  
130 135 140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln Lys  
165 170 175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr  
180 185 190

Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
195 200 205

Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val  
210 215 220

Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile  
225 230 235 240

Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu  
245 250 255

Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln  
260 265 270

Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His Ala  
275 280 285

Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro Gly  
290 295 300

51

ys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys  
 305            310            315            320  
  
 Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn  
 ~            325            330            335  
  
 Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu  
 340            345            350  
  
 Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr  
 355            360            365  
  
 Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu  
 370            375            380  
  
 Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
 385            390            395            400  
  
 Leu

## (2) INFORMATION FOR SEQ ID NO:122:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1324 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 90..1292

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

CCTTATATAAA ACGTCATGAT TGCCTGGGCT GCAGAGACGC ACCTAGCACT GACCCAGCGG  
 60

CTGCCTCCTG AGGTTTCCCG AGGACCACA ATG AAC AAG TGG CTG TGC TGC GCA  
 113

Met Asn Lys Trp Leu Cys Cys Ala  
 1            5

CTC CTG GTG CTC CTG GAC ATC ATT GAA TGG ACA ACC CAG GAA ACC CTT  
 161

Leu Leu Val Leu Leu Asp Ile Ile Glu Trp Thr Thr Gln Glu Thr Leu  
 10            15            20

52

CCT CCA AAG TAC TTG CAT TAT GAC CCA GAA ACT GGT CAT CAG CTC CTG  
209  
Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His Gln Leu Leu  
25           30           35           40

TGT GAC AAA TGT GCT CCT GGC ACC TAC CTA AAA CAG CAC TGC ACA GTG  
257  
Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His Cys Thr Val  
45           50           55

AGG AGG AAG ACA TTG TGT GTC CCT TGC CCT GAC CAC TCT TAT ACG GAC  
305  
Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser Tyr Thr Asp  
60           65           70

AGC TGG CAC ACC AGT GAT GAG TGT GTG TAT TGC AGC CCA GTG TGC AAG  
353  
Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro Val Cys Lys  
75           80           85

GAA CTG CAG TCC GTG AAG CAG GAG TGC AAC CGC ACC CAC AAC CGA GTG  
401  
Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val  
90           95           100

TGT GAG TGT GAG GAA GGG CGT TAC CTG GAG ATC GAA TTC TGC TTG AAG  
449  
Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys  
105           110           115           120

CAC CGG AGC TGT CCC CCG GGC TCC GGC GTG GTG CAA GCT GGA ACC CCA  
497  
His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala Gly Thr Pro  
125           130           135

GAG CGA AAC ACA GTT TGC AAA AAA TGT CCA GAT GGG TTC TTC TCA GGT  
545  
Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe Phe Ser Gly  
140           145           150

GAG ACT TCA TCG AAA GCA CCC TGT ATA AAA CAC ACG AAC TGC AGC ACA  
593  
Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn Cys Ser Thr  
155           160           165

TTT GGC CTC CTG CTA ATT CAG AAA GGA AAT GCA ACA CAT GAC AAC GTG  
641  
Phe Gly Leu Leu Leu Ile Gln Lys Gly Asn Ala Thr His Asp Asn Val  
170           175           180

TGT TCC GGA AAC AGA GAA GCC ACG CAA AAG TGT GGA ATA GAT GTC ACC  
689  
Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile Asp Val Thr

53

185

190

195

200

CTG TGT GAA GAG GCC TTC TTC AGG TTT GCT GTT CCT ACC AAG ATT ATA  
737Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Ile Ile  
205 210 215CCA AAT TGG CTG AGT GTT TTG GTG GAC AGT TTG CCT GGG ACC AAA GTG  
785Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly Thr Lys Val  
220 225 230AAT GCC GAG AGT GTA GAG AGG ATA AAA CGG AGA CAC AGC TCA CAA GAG  
833Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser Ser Gln Glu  
235 240 245CAA ACC TTC CAG CTG CTG AAG CTG TGG AAA CAT CAA AAC AGA GAC CAG  
881Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Arg Asp Gln  
250 255 260GAA ATG GTG AAG AAG ATC ATC CAA GAC ATT GAC CTC TGT GAA AGC AGC  
929Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Ser Ser  
265 270 275 280GTG CAG CGG CAT CTC GGC CAC TCG AAC CTC ACC ACA GAG CAG CTT CTT  
977Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu Gln Leu Leu  
285 290 295GCC TTG ATG GAG AGC CTG CCT GGG AAG AAG ATC AGC CCA GAA GAG ATT  
1025Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro Glu Glu Ile  
300 305 310GAG AGA ACG AGA AAG ACC TGC AAA TCG AGC GAG CAG CTC CTG AAG CTA  
1073Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu Leu Lys Leu  
315 320 325CTC AGT TTA TGG AGG ATC AAA AAT GGT GAC CAA GAC ACC TTG AAG GGC  
1121Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly  
330 335 340CTG ATG TAT GCC CTC AAG CAC TTG AAA ACA TCC CAC TTT CCC AAA ACT  
1169Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe Pro Lys Thr  
345 350 355 360

54

GTC ACC CAC AGT CTG AGG AAG ACC ATG AGG TTC CTG CAC AGC TTC ACA  
1217

Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His Ser Phe Thr  
365 370 375

ATG TAC AGA CTG TAT CAG AAG CTC TTT TTA GAA ATG ATA GGG AAT CAG  
1265

Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn Gln  
380 385 390

GTT CAA TCC GTG AAA ATA AGC TGC TTA TAACTAGGAA TGGTCACTGG  
1312

Val Gln Ser Val Lys Ile Ser Cys Leu  
395 400

GCTGTTTCTT CA 1324

(2) INFORMATION FOR SEQ ID NO:123:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 401 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Leu Leu Asp Ile Ile  
1 5 10 15

Glu Trp Thr Thr Gln Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp  
20 25 30

Pro Glu Thr Gly His Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr  
35 40 45

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro  
50 55 60

Cys Pro Asp His Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys  
65 70 75 80

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu  
85 90 95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr  
100 105 110

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser

55

115 120 125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys  
130 135 140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Ile Lys His Thr Asn Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys  
165 170 175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr  
180 185 190

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
195 200 205

Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val  
210 215 220

Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile  
225 230 235 240

Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu  
245 250 255

Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Ile Ile Gln  
260 265 270

Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Leu Gly His Ser  
275 280 285

Asn Leu Thr Thr Glu Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly  
290 295 300

Lys Lys Ile Ser Pro Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys  
305 310 315 320

Ser Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn  
325 330 335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu  
340 345 350

Lys Thr Ser His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr  
355 360 365

Met Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu  
370 375 380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
385 390 395 400

Leu

## (2) INFORMATION FOR SEQ ID NO:124:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1355 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 94..1296

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

GTATATATAA CGTGATGAGC GTACGGGTGC GGAGACGCAC CGGAGCCGCTC GCCCAGCCGC  
60

CGCTCCAAGC CCCTGAGGTT TCCGGGGACC ACA ATG AAC AAG TTG CTG TGC TGC  
114

Met Asn Lys Leu Leu Cys Cys  
1 5

GCG CTC GTG TTT CTG GAC ATC TCC ATT AAG TGG ACC ACC CAG GAA ACG  
162

Ala Leu Val Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr  
10 15 20

TTT CCT CCA AAG TAC CTT CAT TAT GAC GAA GAA ACC TCT CAT CAG CTG  
210

Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His Gln Leu  
25 30 35

TTG TGT GAC AAA TGT CCT CCT GGT ACC TAC CTA AAA CAA CAC TGT ACA  
258

Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His Cys Thr  
40 45 50 55

GCA AAG TGG AAG ACC GTG TGC GCC CCT TGC CCT GAC CAC TAC TAC ACA  
306

Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr  
60 65 70

GAC AGC TGG CAC ACC AGT GAC GAG TGT CTA TAC TGC AGC CCC GTG TGC  
354

Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys

75            80            85

AAG GAG CTG CAG TAC GTC AAG CAG GAG TGC AAT CGC ACC CAC AAC CGC

402

Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg  
90            95            100GTG TGC GAA TGC AAG GAA GGG CGC TAC CTT GAG ATA GAG TTC TGC TTG  
450Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu  
105            110            115AAA CAT AGG AGC TGC CCT CCT GGA TTT GGA GTG GTG CAA GCT GGA ACC  
498Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr  
120            125            130            135CCA GAG CGA AAT ACA GTT TGC AAA AGA TGT CCA GAT GGG TTC TTC TCA  
546Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe Ser  
140            145            150AAT GAG ACG TCA TCT AAA GCA CCC TGT AGA AAA CAC ACA AAT TGC AGT  
594Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn Cys Ser  
155            160            165GTC TTT GGT CTC CTG CTA ACT CAG AAA GGA AAT GCA ACA CAC GAC AAC  
642Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His Asp Asn  
170            175            180ATA TGT TCC GGA AAC AGT GAA TCA ACT CAA AAA TGT GGA ATA GAT GTT  
690Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile Asp Val  
185            190            195ACC CTG TGT GAG GAG GCA TTC TTC AGG TTT GCT GTT CCT ACA AAG TTT  
738Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Phe  
200            205            210            215ACG CCT AAC TGG CTT AGT GTC TTG GTA GAC AAT TTG CCT GGC ACC AAA  
786Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly Thr Lys  
220            225            230GTA AAC GCA GAG AGT GTA GAG AGG ATA AAA CGG CAA CAC AGC TCA CAA  
834Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser Ser Gln  
235            240            245

GAA CAG ACT TTC CAG CTG CTG AAG TTA TGG AAA CAT CAA AAC AAA GCC  
 882

Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Lys Ala  
 250 255 260

CAA GAT ATA GTC AAG AAG ATC ATC CAA GAT ATT GAC CTC TGT GAA AAC  
 930

Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Asn  
 265 270 275

AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC TTC GAG CAG CTT  
 978

Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu Gln Leu  
 280 285 290 295

CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG GGA GCA GAA GAC  
 1026

Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp  
 300 305 310

ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC CAG ATC CTG AAG  
 1074

Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys  
 315 320 325

CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA GAC ACC TTG AAG  
 1122

Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys  
 330 335 340

GGC CTA ATG CAC GCA CTA AAG CAC TCA AAG ACG TAC CAC TTT CCC AAA  
 1170

Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys  
 345 350 355

ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC CTT CAC AGC TTC  
 1218

Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe  
 360 365 370 375

ACA ATG TAC AAA TTG TAT CAG AAG TTA TTT TTA GAA ATG ATA GGT AAC  
 1266

Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn  
 380 385 390

CAG GTC CAA TCA GTA AAA ATA AGC TGC TTA TAACTGGAAA TGGCCATTGA  
 1316

Gln Val Gln Ser Val Lys Ile Ser Cys Leu  
 395 400

GCTGTTTCCT CACAATTGGC GAGATCCCAG GGATGATAA

1355

## (2) INFORMATION FOR SEQ ID NO:125:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 401 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

Met Asn Lys Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile  
1 5 10 15

Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp  
20 25 30

Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr  
35 40 45

Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro  
50 55 60

Cys Pro Asp His Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys  
65 70 75 80

Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu  
85 90 95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr  
100 105 110

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe  
115 120 125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg  
130 135 140

Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Thr Gln Lys  
165 170 175

Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr  
180 185 190

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
195 200 205

Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val  
210 215 220

60

Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile  
225 230 235 240

Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu  
245 250 255

Trp Lys His Gln Asn Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln  
260 265 270

Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala  
275 280 285

Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly  
290 295 300

Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys  
305 310 315 320

Pro Ser Asp Gln Ile Leu Lys Leu Ser Leu Trp Arg Ile Lys Asn  
325 330 335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser  
340 345 350

Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr  
355 360 365

Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu  
370 375 380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
385 390 395 400

Leu

(2) INFORMATION FOR SEQ ID NO:126:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 139 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys  
1 5 10 15

61

Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro  
20 25 30

Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala  
35 40 45

Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys  
50 55 60

Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr  
65 70 75 80

Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn  
85 90 95

Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His  
100 105 110

Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly  
115 120 125

Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys  
130 135

## (2) INFORMATION FOR SEQ ID NO:127:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 48 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

## (2) INFORMATION FOR SEQ ID NO:128:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 219 amino acids

62

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

x

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala  
1 5 10 15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser  
20 25 30

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Val Glu Thr Gln Asn  
35 40 45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro  
50 55 60

Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro  
65 70 75 80

Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His  
85 90 95

Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly  
100 105 110

Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg  
115 120 125

Cys Lys Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp  
130 135 140

Pro Cys Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr  
145 150 155 160

Ser Asn Thr Lys Cys Lys Glu Glu Gly Ser Arg Ser Asn Leu Gly Trp  
165 170 175

Leu Cys Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg  
180 185 190

Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly  
195 200 205

Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr  
210 215

## (2) INFORMATION FOR SEQ ID NO:129:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 280 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu  
1 5 10 15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro  
20 25 30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys  
35 40 45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys  
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp  
65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu  
85 90 95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val  
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg  
115 120 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe  
130 135 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu  
145 150 155 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu  
165 170 175

Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr  
180 185 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser  
195 200 205

64

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu  
210            215            220

Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys  
225            230            235            240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu  
245            250            255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser  
260            265            270

Phe Ser Pro Thr Pro Gly Phe Thr  
275            280

## (2) INFORMATION FOR SEQ ID NO:130:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 207 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr Val Tyr Gly  
1            5            10            15

Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly Gly His Asp  
20            25            30

Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro Gly Phe Tyr  
35            40            45

Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys Ser Pro Cys  
50            55            60

Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val  
65            70            75            80

Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser Gln Pro Cys  
85            90            95

65

Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly Asn Tyr Cys  
100 105 110

Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro Gln Thr Lys  
115 120 125

\* Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala Gly Asp Thr  
130 135 140

Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser Leu Ser Pro  
145 150 155 160

Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val Gly Phe Asn  
165 170 175

Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Ala Gly His Asn  
180 185 190

Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn Tyr Thr  
195 200 205

(2) INFORMATION FOR SEQ ID NO:131:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 227 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu  
1 5 10 15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr  
20 25 30

Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln  
35 40 45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys  
50 55 60

Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp  
65 70 75 80

Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys  
85 90 95

Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg  
100 105 110

Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu  
~ 115 120 125

Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg  
130 135 140

Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val  
145 150 155 160

Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr  
165 170 175

Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly  
180 185 190

Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser  
195 200 205

Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser  
210 215 220

Gln His Thr  
225

## (2) INFORMATION FOR SEQ ID NO:132:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 197 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

Met Val Ser Leu Pro Arg Leu Cys Ala Leu Trp Gly Cys Leu Leu Thr  
1 5 10 15

Ala Val His Leu Gly Gln Cys Val Thr Cys Ser Asp Lys Gln Tyr Leu  
20 25 30

His Asp Gly Gln Cys Cys Asp Leu Cys Gln Pro Gly Ser Arg Leu Thr  
35 40 45

67

Ser His Cys Thr Ala Leu Glu Lys Thr Gln Cys His Pro Cys Asp Ser  
 50            55            60

Gly Glu Phe Ser Ala Gln Trp Asn Arg Glu Ile Arg Cys His Gln His  
 65            70            75            80

Arg His Cys Glu Pro Asn Gln Gly Leu Arg Val Lys Lys Glu Gly Thr  
 85            90            95

Ala Glu Ser Asp Thr Val Cys Thr Cys Lys Glu Gly Gln His Cys Thr  
 100           105           110

Ser Lys Asp Cys Glu Ala Cys Ala Gln His Thr Pro Cys Ile Pro Gly  
 115           120           125

Phe Gly Val Met Glu Met ala Thr Glu Thr Thr Asp Thr Val Cys His  
 130           135           140

Pro Cys Pro Val Gly Phe Phe Ser Asn Gln Ser Ser Leu Phe Glu Lys  
 145           150           155           160

Cys Tyr Pro Trp Thr Ser Cys Glu Asp Lys Asn Leu Glu Val Leu Gln  
 165           170           175

Lys Gly Thr Ser Gln Thr Asn Val Ile Cys Gly Leu Lys Ser Arg Met  
 180           185           190

Arg Ala Leu Leu Val  
 195

## (2) INFORMATION FOR SEQ ID NO:133:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 208 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile  
 1            5            10            15

Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp  
 20           25            30

Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr  
 35           40            45

68

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro  
50 55 60

Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys  
65 70 75 80

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu  
85 90 95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr  
100 105 110

Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu  
115 120 125

Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg  
130 135 140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln Lys  
165 170 175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr  
180 185 190

Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Ala Phe Phe Arg  
195 200 205

## (2) INFORMATION FOR SEQ ID NO:134:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 224 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

Met Gly Ala Gly Ala Thr Gly Arg Ala Met Asp Gly Pro Arg Leu Leu  
1 5 10 15

Leu Leu Leu Leu Leu Gly Val Ser Leu Gly Gly Ala Lys Glu Ala Cys  
20 25 30

Pro Thr Gly Leu Tyr Thr His Ser Gly Glu Cys Cys Lys Ala Cys Asn  
35 40 45

69

Leu Gly Glu Gly Val Ala Gln Pro Cys Gly Ala Asn Gln Thr Val Cys  
50            55            60

Glu Pro Cys Leu Asp Ser Val Thr Phe Ser Asp Val Val Ser Ala Thr  
65            70            75            80

Glu Pro Cys Lys Pro Cys Thr Glu Cys Val Gly Leu Gln Ser Met Ser  
85            90            95

Ala Pro Cys Val Glu Ala Asp Asp Ala Val Cys Arg Cys Ala Tyr Gly  
100            105            110

Tyr Tyr Gln Asp Glu Thr Thr Gly Arg Cys Glu Ala Cys Arg Val Cys  
115            120            125

Glu Ala Gly Ser Gly Leu Val Phe Ser Cys Gln Asp Lys Gln Asn Thr  
130            135            140

Val Cys Glu Glu Cys Pro Asp Gly Thr Tyr Ser Asp Glu Ala Asn His  
145            150            155            160

Val Asp Pro Cys Leu Pro Cys Thr Val Cys Glu Asp Thr Glu Arg Gln  
165            170            175

Leu Arg Glu Cys Thr Arg Trp Ala Asp Ala Glu Cys Glu Glu Ile Pro  
180            185            190

Gly Arg Trp Ile Thr Arg Ser Thr Pro Pro Glu Gly Ser Asp Ser Thr  
195            200            205

Ala Pro Ser Thr Gln Glu Pro Glu Ala Pro Pro Glu Gln Asp Leu Ile  
210            215            220

## (2) INFORMATION FOR SEQ ID NO:135:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 205 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

Met Tyr Val Trp Val Gln Gln Pro Thr Ala Phe Leu Leu Gly Leu  
1            5            10            15

Ser Leu Gly Val Thr Val Lys Leu Asn Cys Val Lys Asp Thr Tyr Pro  
20            25            30

70

Ser Gly His Lys Cys Cys Arg Glu Cys Gln Pro Gly His Gly Met Val  
35            40            45

Ser Arg Cys Asp His Thr Arg Asp Thr Val Cys His Pro Cys Glu Pro  
50            55            60

Gly Phe Tyr Asn Glu Ala Val Asn Tyr Asp Thr Cys Lys Gln Cys Thr  
65            70            75            80

Gln Cys Asn His Arg Ser Gly Ser Glu Leu Lys Gln Asn Cys Thr Pro  
85            90            95

Thr Glu Asp Thr Val Cys Gln Cys Arg Pro Gly Thr Gln Pro Arg Gln  
100            105            110

Asp Ser Ser His Lys Leu Gly Val Asp Cys Val Pro Cys Pro Pro Gly  
115            120            125

His Phe Ser Pro Gly Ser Asn Gln Ala Cys Lys Pro Trp Thr Asn Cys  
130            135            140

Thr Leu Ser Gly Lys Gln Ile Arg His Pro Ala Ser Asn Ser Leu Asp  
145            150            155            160

Thr Val Cys Glu Asp Arg Ser Leu Leu Ala Thr Leu Leu Trp Glu Thr  
165            170            175

Gln Arg Thr Thr Phe Arg Pro Thr Thr Val Pro Ser Thr Thr Val Trp  
180            185            190

Pro Arg Thr Ser Gln Leu Pro Ser Thr Pro Thr Leu Val  
195            200            205

(2) INFORMATION FOR SEQ ID NO:136:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 191 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

Met Gly Asn Asn Cys Tyr Asn Val Val Val Ile Val Leu Leu Val  
1            5            10            15

71

Gly Cys Glu Lys Val Gly Ala Val Gln Asn Ser Cys Asp Asn Cys Gln  
20 25 30

Pro Gly Thr Phe Cys Arg Lys Tyr Asn Pro Val Cys Lys Ser Cys Pro  
35 40 45

Pro Ser Thr Phe Ser Ser Ile Gly Gly Gln Pro Asn Cys Asn Ile Cys  
50 55 60

Arg Val Cys Ala Gly Tyr Phe Arg Phe Lys Lys Phe Cys Ser Ser Thr  
65 70 75 80

His Asn Ala Glu Cys Glu Cys Ile Glu Gly Phe His Cys Leu Gly Pro  
85 90 95

Gln Cys Thr Arg Cys Glu Lys Asp Cys Arg Pro Gly Gln Glu Leu Thr  
100 105 110

Lys Gln Gly Cys Lys Thr Cys Ser Leu Gly Thr Phe Asn Asp Gln Asn  
115 120 125

Gly Thr Gly Val Cys Arg Pro Trp Thr Asn Cys Ser Leu Asp Gly Arg  
130 135 140

Ser Val Leu Lys Thr Gly Thr Thr Glu Lys Asp Val Val Cys Gly Pro  
145 150 155 160

Pro Val Val Ser Phe Ser Pro Ser Thr Thr Ile Ser Val Thr Pro Glu  
165 170 175

Gly Gly Pro Gly Gly His Ser Leu Gln Val Leu Thr Leu Phe Leu  
180 185 190

## (2) INFORMATION FOR SEQ ID NO:137:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTAC  
54

## (2) INFORMATION FOR SEQ ID NO:138:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 380 amino acids

(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

\*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His  
1 5 10 15

Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His  
20 25 30

Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser  
35 40 45

Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro  
50 55 60

Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His  
65 70 75 80

Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe  
85 90 95

Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala  
100 105 110

Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe  
115 120 125

Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn  
130 135 140

Cys Ser Thr Phe Gly Leu Leu Ile Gln Lys Gly Asn Ala Thr His  
145 150 155 160

Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile  
165 170 175

Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr  
180 185 190

Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly  
195 200 205

Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser  
210 215 220

Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn

73

225	230	235	240
Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys			
245	250	255	
Glu Ser Ser Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu			
260	265	270	
Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro			
275	280	285	
Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu			
290	295	300	
Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr			
305	310	315	320
Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe			
325	330	335	
Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His			
340	345	350	
Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile			
355	360	365	
Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu			
370	375	380	

## (2) INFORMATION FOR SEQ ID NO:139:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 380 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

1	5	10	15
---	---	----	----

20	25	30	
----	----	----	--

35	40	45	
----	----	----	--

Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro  
50 55 60

Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His  
65 70 75 80

Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe  
85 90 95

Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala  
100 105 110

Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe  
115 120 125

Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn  
130 135 140

Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His  
145 150 155 160

Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile  
165 170 175

Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr  
180 185 190

Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly  
195 200 205

Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser  
210 215 220

Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn  
225 230 235 240

Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys  
245 250 255

Glu Asn Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu  
260 265 270

Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala  
275 280 285

Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile  
290 295 300

Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr  
305 310 315 320

Leu Lys Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe

75

325                  330                  335

Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His  
340                  345                  350

Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile  
355                  360                  365

Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu  
370                  375                  380

## (2) INFORMATION FOR SEQ ID NO:140:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

TGGACCACCC AGAAGTACCT TCATTATGAC                  30

## (2) INFORMATION FOR SEQ ID NO:141:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

GTCATAATGA AGGTACTTCT GGGTGGTCCA                  30

## (2) INFORMATION FOR SEQ ID NO:142:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

76

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

GGACCACCCA GCTTCATTAT GACGAAGAAA C 31

## (2) INFORMATION FOR SEQ ID NO:143:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

GTTTCTTCGT CATAATGAAG CTGGGTGGTC C 31

## (2) INFORMATION FOR SEQ ID NO:144:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

GTGGACCACC CAGGACGAAG AAACCTCTC 29

## (2) INFORMATION FOR SEQ ID NO:145:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:

GAGAGGTTTC TTCGTCTGG GTGGTCCAC 29

77

## (2) INFORMATION FOR SEQ ID NO:146:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:

CGTTTCCTCC AAAGTTCCCTT CATTATGAC

29

## (2) INFORMATION FOR SEQ ID NO:147:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:

GTCATAATGA AGGAACTTTG GAGGAAACG

29

## (2) INFORMATION FOR SEQ ID NO:148:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:

GGAAACGTTT CCTGCAAAGT ACCTTCATTA TG

32

## (2) INFORMATION FOR SEQ ID NO:149:

78

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:

CATAATGAAG GTACTTGCA GGAAACGTTT CC

32

## (2) INFORMATION FOR SEQ ID NO:150:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:

CACGCAAAAG TCGGGAATAG ATGTCAC

27

## (2) INFORMATION FOR SEQ ID NO:151:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:

GTGACATCTA TTCCCGACTT TTGCGTG

27

## (2) INFORMATION FOR SEQ ID NO:152:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid

79

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

\*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:

CACCCTGTCTG GAAGAGGCCT TCTTC

25

(2) INFORMATION FOR SEQ ID NO:153:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:153:

GAAGAAGGCC TCTTCCGACA GGGTG

25

(2) INFORMATION FOR SEQ ID NO:154:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:

TGACCTCTCG GAAAGCAGCG TGCA

24

(2) INFORMATION FOR SEQ ID NO:155:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:

TGCACGCTGC TTTCCGAGAG GTCA 24

(2) INFORMATION FOR SEQ ID NO:156:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:

CCTCGAAATC GAGCGAGCAG CTCC 24

(2) INFORMATION FOR SEQ ID NO:157:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:

CGATTCGAG GTCTTCTCG TTCTC 25

(2) INFORMATION FOR SEQ ID NO:158:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:

CCGTGAAAAT AAGCTCGTTA TAACTAGGAA TGG

33

(2)\* INFORMATION FOR SEQ ID NO:159:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:

CCATTCCTAG TTATAACGAG CTTATTTCA CGG

33

(2) INFORMATION FOR SEQ ID NO:160:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:160:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:161:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:161:

CCTCTCTCGA GTCAGGTGAC ATCTATTCCA CACTTTGCG TGGC

44

## (2) INFORMATION FOR SEQ ID NO:162:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:162:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:163:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:163:

CCTCTCTCGA GTCAAGGAAC AGCAAACCTG AAGAAGGC

38

## (2) INFORMATION FOR SEQ ID NO:164:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:164:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:165:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:165:

CCTCTCTCGA GTCACTCTGT GGTGAGGTTTC GAGTGGCC

38

## (2) INFORMATION FOR SEQ ID NO:166:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:166:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:167:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:167:

CCTCTCTCGA GTCAGGATGT TTTCAAGTGC TTGAGGGC

38

## (2) INFORMATION FOR SEQ ID NO:168:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids

84

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

\*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:168:

Met Lys His His His His His Ala Ser Val Asn Ala Leu Glu  
1 5 10 15

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



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(10) International Publication Number  
**WO 01/03719 A3**

(51) International Patent Classification<sup>7</sup>: **A61K 38/17.**  
38/20, 38/55, 47/48, A61P 19/00, 25/00, 29/00, 37/00 //  
C07K 14/705, C12N 15/12, (A61K 38/17, 38:20) (A61K  
38/17, 38:55) (A61K 38/17, 38:20, 38:55) (A61K 38/20,  
38:55)

(21) International Application Number: PCT/US00/18667

(22) International Filing Date: 7 July 2000 (07.07.2000)

(25) Filing Language: English

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(30) Priority Data:  
09/350,670 9 July 1999 (09.07.1999) US  
09/457,647 9 December 1999 (09.12.1999) US

(71) Applicant: AMGEN INC. [US/US]; One Amgen Center Drive, Thousand Oaks, CA 91320-1799 (US).

(72) Inventors: BOYLE, William, J.; 11678 Chestnut Ridge Street, Moorpark, CA 93021 (US). LACEY, David, Lee; 614 Paseo Vista, Newbury Park, CA 91320 (US). CALZONE, Frank, J.; 841 Pine Crest Circle, Westlake Village, CA 91361 (US). CHANG, Ming-Shi; 3rd Floor, No.58 Tong-Ning Road, Tainan (TW). SENALDI, Giorgio; 2846 White Ridge Place, Thousand Oaks, CA 91362 (US).

(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., One Amgen Center Drive, 274-A, Thousand Oaks, CA 91320-1799 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report

(88) Date of publication of the international search report:  
21 February 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/03719 A3

(54) Title: COMBINATION THERAPY FOR CONDITIONS LEADING TO BONE LOSS

(57) Abstract: The present invention discloses a novel secreted polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily and is involved in the regulation of bone metabolism. Also disclosed are nucleic acids encoding osteoprotegerin, polypeptides, recombinant vectors and host cells for expression, antibodies which bind OPG, and pharmaceutical compositions. The polypeptides are used to treat bone diseases characterized by increased resorption such as osteoporosis. Methods of treatment are described using the polypeptides in conjunction with various agents, including IL-1 inhibitors, TNF- $\alpha$  inhibitors, and serine protease inhibitors.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/18667

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/17 A61K38/20 A61K38/55 A61K47/48 A61P19/00  
 A61P25/00 A61P29/00 A61P37/00 //C07K14/705,  
 C12N15/12, (A61K38/17, 38:20), (A61K38/17, 38:55), (A61K38/17, 38:20),

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE, SCISEARCH, EMBASE, CANCERLIT

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 784 093 A (AMGEN INC) 16 July 1997 (1997-07-16) cited in the application  abstract page 12, line 41 - line 49; claims 1-60 ---	1,2, 6-13, 17-25, 30-42
A	ROODMAN G D: "ROLE OF CYTOKINES IN THE REGULATION OF BONE RESORPTION" CALCIFIED TISSUE INTERNATIONAL, NEW YORK, NY, US, vol. 53, no. SUPPL. 01, 1993, pages S94-S98, XP000940463 ISSN: 0171-967X page S95, left-hand column, paragraph 3 -right-hand column, paragraph 1 --- -/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

24 January 2001

12.07.2001

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

NIEMANN, F

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/18667

**A. CLASSIFICATION OF SUBJECT MATTER**  
**IPC 7 38:55), (A61K38/20, 38:55)**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 93 21946 A (RUSSELL DEBORAH A ; THOMPSON ROBERT C (US); SYNERGEN INC (US))            11 November 1993 (1993-11-11)            abstract            claims 1-15</p> <p>---</p> <p>MARTIN T J ET AL: "Interleukins in the control of osteoclast differentiation." CRITICAL REVIEWS IN EUKARYOTIC GENE EXPRESSION, (1998) 8 (2) 107-23. REF: 143, XP000979080            the whole document</p> <p>-----</p>	
A		

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

24 January 2001

Name and mailing address of the ISA

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 Fax: (+31-70) 340-3016

Authorized officer

NIEMANN, F

## INTERNATIONAL SEARCH REPORT

I national application No.  
PCT/US 00/18667

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  

Although claims 1,2,6-13,17-25,30-42 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see further information sheet in evention 1.

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,2,39-42 (partially), 6-13,17-25,30-38

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with TNF-alpha inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with TNF-alpha inhibitors and IL-1 inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with TNF-alpha inhibitors and serine protease inhibitors.

2. Claims: 1,2,39-42 (partially) 14-16, 26-29

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors and IL-1 inhibitors.

3. Claims: 1,2 (partially),3-5

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors and IL-1 inhibitors.

4. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with IL-6 inhibitors.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with IL-8 inhibitors.

6. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with IL-18 inhibitors.

7. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with ICE modulators.

8. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with FGF-1 to FGF-10, FGF modulators.

9. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with PAF antagonists.

10. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with MMP modulators.

11. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with NOS modulators.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

12. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with modulators of glucocorticoid receptor.

13. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with modulators of glutamate receptor.

14. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with modulators of LPS level.

15. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with noradrenaline or modulators and mimetics thereof.

16. Claims: 43-48

a method of treating an IL-1 mediated disease, which comprises administering therapeutically effective amounts of an IL-1 inhibitor and a serine protease inhibitor

17. Claims: 49-57

a method of treating an TNF-mediated disease, which comprises administering therapeutically effective amounts of a TNF-alpha inhibitor and a serine protease inhibitor

18. Claims: 58-61

a method of treating inflammation, rheumatoid arthritis, SLE, GvHD, which comprises administering an IL-18 inhibitor, a TNF-alpha inhibitor, and an IL-1 inhibitor

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/18667

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0784093	A 16-07-1997	AU	710587 B	23-09-1999
		AU	1468697 A	17-07-1997
		BG	101813 A	30-09-1998
		CA	2210467 A	03-07-1997
		CN	1182452 A	20-05-1998
		CZ	9702538 A	17-03-1999
		DE	19654610 A	26-06-1997
		EP	0870023 A	14-10-1998
		FR	2742767 A	27-06-1997
		GB	2312899 A, B	12-11-1997
		HU	9801122 A	28-08-1998
		JP	11503616 T	30-03-1999
		NO	973699 A	21-10-1997
		NZ	326579 A	28-01-1999
		PL	321938 A	05-01-1998
		SK	110797 A	12-07-1999
		TR	970550 A	21-07-1997
		WO	9723614 A	03-07-1997
		US	6015938 A	18-01-2000
<hr/>				
WO 9321946	A 11-11-1993	AT	188610 T	15-01-2000
		AU	4229493 A	29-11-1993
		CA	2118119 A	11-11-1993
		DE	69327582 D	17-02-2000
		DE	69327582 T	03-08-2000
		DK	639079 T	13-06-2000
		EP	0639079 A	22-02-1995
		ES	2142341 T	16-04-2000
		GR	3033144 T	31-08-2000
		JP	7509223 T	12-10-1995
		PT	639079 T	28-04-2000
<hr/>				

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International Bureau



(43) International Publication Date  
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PCT

(10) International Publication Number  
**WO 01/003719 A3**

(51) International Patent Classification<sup>7</sup>: **A61K 38/17, 38/20, 38/55, 47/48, A61P 19/00, 25/00, 29/00, 37/00 // C07K 14/705, C12N 15/12, (A61K 38/17, 38:20) (A61K 38/17, 38:55) (A61K 38/17, 38:20, 38:55) (A61K 38/20, 38:55)**

(21) International Application Number: PCT/US00/18667

(22) International Filing Date: 7 July 2000 (07.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/350,670 9 July 1999 (09.07.1999) US  
09/457,647 9 December 1999 (09.12.1999) US



(71) Applicant: AMGEN INC. [US/US]; One Amgen Center Drive, Thousand Oaks, CA 91320-1799 (US).

(72) Inventors: BOYLE, William, J.; 11678 Chestnut Ridge Street, Moorpark, CA 93021 (US). LACEY, David, Lee; 614 Paseo Vista, Newbury Park, CA 91320 (US). CAL-ZONE, Frank, J.; 841 Pine Crest Circle, Westlake Village, CA 91361 (US). CHANG, Ming-Shi; 3rd Floor, No.58 Tong-Ning Road, Tainan (TW). SENALDI, Giorgio; 2846 White Ridge Place, Thousand Oaks, CA 91362 (US).

(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., One Amgen Center Drive, 27-4-A, Thousand Oaks, CA 91320-1799 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report

(88) Date of publication of the international search report: 21 February 2002

(48) Date of publication of this corrected version: 8 August 2002

(15) Information about Correction:

see PCT Gazette No. 32/2002 of 8 August 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**WO 01/003719 A3**

(54) Title: COMBINATION THERAPY FOR CONDITIONS LEADING TO BONE LOSS

(57) Abstract: The present invention discloses a novel secreted polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily and is involved in the regulation of bone metabolism. Also disclosed are nucleic acids encoding osteoprotegerin, polypeptides, recombinant vectors and host cells for expression, antibodies which bind OPG, and pharmaceutical compositions. The polypeptides are used to treat bone diseases characterized by increased resorption such as osteoporosis. Methods of treatment are described using the polypeptides in conjunction with various agents, including IL-1 inhibitors, TNF- $\alpha$  inhibitors, and serine protease inhibitors.

**COMBINATION THERAPY FOR  
CONDITIONS LEADING TO BONE LOSS**

**Cross-Reference to Related Applications**

5        This application is a continuation-in-part of U.S. Ser. No. 09/350,670 filed July 9, 1999, which is a continuation-in-part (CIP) of U.S. Ser. No. 08/706,945, filed on September 3, 1996, which in turn is a CIP of U.S. Ser. No. 08/577,788, filed December 22, 1995. Each 10 of the foregoing applications is hereby incorporated by reference.

**Field of the Invention**

15      The invention relates generally to polypeptides involved in the regulation of bone metabolism. More particularly, the invention relates to a novel polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily. The polypeptide is used to treat bone diseases characterized by increased bone loss such as 20 osteoporosis and arthritis.

**Background of the Invention**

25      Polypeptide growth factors and cytokines are secreted factors which signal a wide variety of changes in cell growth, differentiation, and metabolism, by specifically binding to discrete, surface bound receptors. As a class of proteins, receptors vary in their structure and mode of signal transduction. They are characterized by having an extracellular domain that is involved in ligand binding, and cytoplasmic 30 domain which transmits an appropriate intracellular signal. Receptor expression patterns ultimately determine which cells will respond to a given ligand, while the structure of a given receptor dictates the cellular response induced by ligand binding. Receptors 35 have been shown to transmit intracellular signals via

their cytoplasmic domains by activating protein tyrosine, or protein serine/threonine phosphorylation (e.g., platelet derived growth factor receptor (PDGFR) or transforming growth factor- $\beta$  receptor-I (TGF $\beta$ R-I),

- 5 by stimulating G-protein activation (e.g.,  $\beta$ -adrenergic receptor), and by modulating associations with cytoplasmic signal transducing proteins (e.g., TNFR-I and Fas/APO) (Heldin, *Cell* 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, *et al.* *Cell* 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of these receptors (Chen *et al.*, *Chemistry* 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNF $\alpha$ . (Goeddel *et al.* *Cold Spring Harbor Symp. Quart. Biol.* 51, 597-609 (1986); Nagata *et al.* *Science* 267, 1449-1456 (1995)). TNF $\alpha$  binds to distinct, but closely related receptors, TNFR-I and TNFR-II. TNF $\alpha$  produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler *et al.* *Ann. Rev. Biochem.* 57, 505-518 (1988)).

TNF $\alpha$  is believed to mediate acute and chronic inflammatory responses (Beutler *et al.* *Ann. Rev. Biochem.* 57, 505-508 (1988)). Systemic delivery of TNF $\alpha$  induces toxic shock and widespread tissue necrosis. Because of this, TNF $\alpha$  may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda *et al.* *Cell* 75, 1169-1178 (1993)), is associated

with autoimmunity (Fisher *et al.* *Cell* 81, 935-946 (1995)), while overproduction of FasL may be implicated in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects 5 of many disease states, which suggests that agents that neutralize the activity of these ligands would have therapeutic value. Soluble TNFR-I receptors, and antibodies that bind TNF $\alpha$ , have been tested for their ability to neutralize systemic TNF $\alpha$  (Loetscher *et al.* 10 *Cancer Cells* 3(6), 221-226 (1991)). A naturally occurring form of a secreted TNFR-I mRNA was cloned, and its product tested for its ability to neutralize TNF $\alpha$  activity *in vitro* and *in vivo* (Kohno *et al.* *PNAS USA* 87, 8331-8335 (1990)). The ability of this protein 15 to neutralize TNF $\alpha$  suggests that soluble TNF receptors function to bind and clear TNF thereby blocking the cytotoxic effects on TNFR- bearing cells.

An object of the invention is to identify new members of the TNFR superfamily. It is anticipated that 20 new family members may be transmembrane proteins or soluble forms thereof comprising extracellular domains and lacking transmembrane and cytoplasmic domains. We have identified a new member of the TNFR superfamily which encodes a secreted protein that is closely 25 related to TNFR-II. By analogy to soluble TNFR-II, the TNFR-II related protein may negatively regulate the activity of its ligand, and thus may be useful in the treatment of certain human diseases.

A further object of this invention is new methods 30 of treatment of inflammatory diseases and medical conditions.

#### Summary of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily has been identified from a 35 fetal rat intestinal cDNA library. A full-length cDNA

clone was obtained and sequenced. Expression of the rat cDNA in a transgenic mouse revealed a marked increase in bone density, particularly in long bones, pelvic bone and vertebrae. The polypeptide encoded by the cDNA 5 is termed Osteoprotegerin (OPG) and plays a role in promoting bone accumulation.

The invention provides for nucleic acids encoding a polypeptide having at least one of the biological activities of OPG. Nucleic acids which hybridize to 10 nucleic acids encoding mouse, rat or human OPG as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO: 122), and 9C-9D (SEQ ID NO: 124) are also provided. Preferably, OPG is mammalian OPG and more preferably is 15 human OPG. Recombinant vectors and host cells expressing OPG are also encompassed as are methods of producing recombinant OPG. Antibodies or fragments thereof which specifically bind the polypeptide are also disclosed.

Methods of treating bone diseases are also 20 provided by the invention. The polypeptides are useful for preventing bone resorption and may be used to treat any condition resulting in bone loss such as osteoporosis, hypercalcemia, Paget's disease of bone, and bone loss due to rheumatoid arthritis or 25 osteomyelitis, and the like. Bone diseases may also be treated with anti-sense or gene therapy using nucleic acids of the invention. Pharmaceutical compositions comprising OPG nucleic acids and polypeptides are also encompassed.

30 The invention relates further to treatment of diseases using combination therapy. In particular, the novel polypeptides described herein may be used in conjunction with bone morphogenic proteins BMP-1 through BMP-12; TGF- $\beta$  and TGF- $\beta$  family members; IL-1 35 inhibitors; TNF- $\alpha$  inhibitors; parathyroid hormone and

analogs thereof; parathyroid related protein and  
analogs thereof; E series prostaglandins;  
bisphosphonates; bone-enhancing minerals; NSAIDs;  
immunosuppressants; serine protease inhibitors; IL-6  
5 inhibitors; IL-8 inhibitors (e.g., antibodies to IL-8);  
IL-18 inhibitors; ICE modulators; FGF-1 to FGF-10; FGF  
modulators; PAF antagonists; KGF, KGF-related  
molecules, or KGF modulators; MMP modulators; NOS  
modulators; modulators of glucocorticoid receptor;  
10 modulators of glutamate receptor; modulators of LPS  
levels; and noradrenaline and modulators and mimetics  
thereof.

**Description of the Figures**

Figure 1. A. FASTA analysis of novel EST LORF.  
15 Shown is the deduced FRI-1 amino acid sequence aligned  
to the human TNFR-II sequence. B. Profile analysis of  
the novel EST LORF shown is the deduced FRI-1 amino  
acid sequence aligned to the TNFR-profile. C.  
Structural view of TNFR superfamily indicating region  
20 which is homologous to the novel FRI-1.

Figure 2. Structure and sequence of full length  
rat OPG gene, a novel member of the TNFR superfamily.  
A. Map of pMOB-B1.1 insert. Box indicates position of  
LORF within the cDNA sequence (bold line). Black box  
25 indicates signal peptide, and gray ellipses indicate  
position of cysteine-rich repeat sequences. B, C.  
Nucleic acid and protein sequence of the Rat OPG cDNA.  
The predicted signal peptide is underlined, and  
potential sites of N-linked glycosylation are indicated  
30 in bold, underlined letters. D, E. Pileup sequence  
comparison (Wisconsin GCG Package, Version 8.1) of OPG  
with other members of the TNFR superfamily, fas (SEQ ID  
NO:128); tnfr1 (SEQ ID NO: 129); sfu-t2 (SEQ ID  
NO:130); tnfr2 (SEQ ID NO:131); cd40 (SEQ ID NO:132);  
35 osteo (SEQ ID NO:133); ngfr (SEQ ID NO:134); ox40 (SEQ  
ID NO:135); 41bb (SEQ ID NO:136).

Figure 3. PepPlot analysis (Wisconsin GCG Package, Version 8.1) of the predicted rat OPG sequence. A. Schematic representation of rat OPG showing hydrophobic (up) and hydrophilic (down) amino acids. Also shown are 5 basic (up) and acidic (down) amino acids. B. Display of amino acid residues that are beta-sheet forming (up) and beta-sheet breaking (down) as defined by Chou and Fasman (Adv. Enz. 47, 45-147 (1948)). C. Display of propensity measures for alpha-helix and beta-sheet 10 (Chou and Fasman, ibid). Curves above 1.00 show propensity for alpha-helix or beta-sheet structure. Structure may terminate in regions of protein where curves drop below 1.00. D. Display of residues that are alpha-forming (up) or alpha-breaking (down). E. Display 15 of portions of the protein sequence that resemble sequences typically found at the amino end of alpha and beta structures (Chou and Fasman, ibid). F. Display of portions of the protein sequence that resemble sequences typically found at the carboxyl end of alpha 20 and beta structures (Chou and Fasman, ibid). G. Display of portions of the proteins sequence typically found in turns (Chou and Fasman, ibid) H. Display of the helical hydrophobic moment (Eisenberg et al. Proc. Natl. Acad. Sci. USA 81, 140-144 (1984)) at each position in the 25 sequence. I. Display of average hydrophathy based upon Kyte and Doolittle (J. Mol. Biol. 157, 105-132 (1982)) and Goldman et al. (reviewed in Ann. Rev. Biophys. Biophys. Chem. 15, 321-353 (1986)).

Figure 4. mRNA expression patterns for the OPG 30 cDNA in human tissues. Northern blots were probed with a 32P-labeled rat cDNA insert (A, left two panels), or with the human cDNA insert (B, right panel).

Figure 5. Creation of transgenic mice expressing the OPG cDNA in hepatocytes. Northern blot expression 35 of HE-OPG transgene in mouse liver.

Figure 6. Increase in bone density in OPG transgenic mice. Panel A-F. Control Mice. G-J, OPG expressing mice. At necropsy, all animals were radiographed and photographs prepared. In A-F, the 5 radiographs of the control animals and the one transgenic non-expressor (#28) are shown. Note that the bones have a clearly defined cortex and a lucent central marrow cavity. In contrast, the OPG (G-J) animals have a poorly defined cortex and increased 10 density in the marrow zone.

Figure 7. Increase in trabecular bone in OPG transgenic mice. A-D. Representative photomicrographs of bones from control animals. In A and B, low (4X, 10X) power images of the femurs are shown (Masson 15 Trichrome stain). Stains for tartrate resistant acid phosphatase (TRAP) demonstrate osteoclasts (see arrows) both resorbing cartilage (C) and trabecular bone (D). Note the flattened appearance of osteoclasts on trabecular bone. E-H. Representative photomicrographs 20 of bones from OPG-expressing animals. In E and F, low (4X, 10X) power images of the femurs are shown (Masson Trichrome stain). The clear region is the growth plate cartilage, blue stained area is bone, and the red area is marrow. Note that in contrast to the controls, the 25 trabecular bone has not been resorbed resulting in the absence of the usual marrow cavity. Also, the resulting trabeculae have a variegated appearance with blue and clear areas. The clear areas are remnants of growth plate cartilage that have never been remodelled. Based 30 on TRAP stains, these animals do have osteoclasts (see arrows) at the growth plate (G), which may be reduced in number. However, the surfaces of the trabeculae away from the growth plate are virtually devoid of 35 osteoclasts (H), a finding that stands in direct contrast with the control animals (see D).

Figure 8. HE-OPG expressors do not have a defect in monocyte-macrophage development. One cause for osteopetrosis in mice is defective M-CSF production due to a point mutation in the M-CSF gene. This results in 5 a marked deficit of circulating and tissue based macrophages. The peripheral blood of OPG expressors contained monocytes as assessed by H1E analysis. To affirm the presence of tissue macrophages, immnohistochemistry was performed using F480 10 antibodies, which recognize a cell surface antigen on murine macrophages. A and C show low power (4X) photomicrographs of the spleens from normal and CR1 overexpressors. Note that both animals have numerous F480 positive cells. Monocyte-macrophages were also 15 present in the marrow of normal (B) and HE-OPG overexpressors (D) (40X).

Figure 9. Structure and sequence of mouse and human OPG cDNA clones. A, B. Mouse cDNA and protein sequence. C, D. Human cDNA and protein sequence. The 20 predicted signal peptides are underlined, and potential sites of N-linked glycosylation are indicated in bold. E, F. Sequence alignment and comparison of rat, mouse and human OPG amino acid sequences.

Figure 10. Comparison of conserved sequences in 25 extracellular domain of TNFR-I and human OPG. PrettyPlot (Wisconsin GCG Package, Version 8.1) of the TNFR1 and OPG alignment described in example 6. Top line, human TNFR1 sequences encoding domains 1-4. Bottom line, human OPG sequences encoding domains 1-4. 30 Conserved residues are highlighted by rectangular boxes.

Figure 11. Three-dimensional representation of 35 human OPG. Side-view of the Molescript display of the predicted 3-dimensional structure of human OPG residues 25 through 163, (wide line), co-crystallized with human

TNF $\beta$  (thin line). As a reference for orientation, the bold arrows along the OPG polypeptide backbone are pointing in the N-terminal to C-terminal direction. The location of individual cysteine residue side chains are 5 inserted along the polypeptide backbone to help demonstrate the separate cysteine-rich domains. The TNF $\beta$  molecule is aligned as described by Banner *et al.* (1993).

Figure 12. Structure of OPG cysteine-rich domains.  
10 Alignment of the human (top line SEQ ID NO:136) and mouse (bottom line) OPG amino acid sequences highlighting the predicted domain structure of OPG. The polypeptide is divided into two halves; the N-terminus (A), and C-terminus (B). The N-terminal half is 15 predicted to contain four cysteine rich domains (labeled 1-4). The predicted intrachain disulfide bonds are indicated by bold lines, labeled "SS1", "SS2", or "SS3". Tyrosine 28 and histidine 75 (underlined) are predicted to form an ionic interaction. Those amino acids predicted to interact with an OPG ligand are 20 indicated by bold dots above the appropriate residue. The cysteine residues located in the C-terminal half of OPG are indicated by rectangular boxes.

Figure 13. Expression and secretion of full length 25 and truncated mouse OPG-Fc fusion proteins. A. Map indicating points of fusion to the human IgG1 Fc domain are indicated by arrowheads. B. Silver stain of a SDS-polyacrylamide gel of conditioned media obtained from cells expressing either Fl.Fc (Full length OPG fused to 30 Fc at Leucine 401) or CT.Fc (Carboxy-terminal truncated OPG fused to Fc at threonine 180) fusion protein expression vectors. Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line. C. Western blot of 35 conditioned media obtained from Fl.Fc and CT.Fc fusion

protein expression vectors probed with anti-human IgG1 Fc domain (Pierce). Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line.

5       Figure 14. Expression of human OPG in E. coli. A. Construction of a bacterial expression vector. The LORF of the human OPG gene was amplified by PCR, then joined to a oligonucleotide linker fragment (top strand is SEQ ID NO:137; bottom strand is SEQ ID NO:127), and ligated  
10      into pAMG21 vector DNA. The resulting vector is capable of expressing OPG residues 32-401 linked to a N-terminal methionine residue. B SDS-PAGE analysis of uninduced and induced bacterial harboring the pAMG21-human OPG -32-401 plasmid. Lane 1, MW standards; lane  
15      2, uninduced bacteria; lane 3, 30°C induction; lane 4, 37°C induction; lane 5, whole cell lysate from 37°C induction; lane 6, soluble fraction of whole cell lysate; lane 7, insoluble fraction of whole cell lysate; lane 8, purified inclusion bodies obtained from  
20      whole cell lysate.

Figure 15. Analysis of recombinant murine OPG produced in CHO cells by SDS-PAGE and western blotting. An equal amount of CHO conditioned media was applied to each lane shown, and was prepared by treatment with  
25      either reducing sample buffer (left lane), or non-reducing sample buffer (right lane). After electrophoresis, the resolved proteins were transferred to a nylon membrane, then probed with anti-OPG antibodies. The relative positions of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.  
30

Figure 16. Pulse-chase analysis of recombinant murine OPG produced in CHO cells. CHO cells were pulse-labeled with  $^{35}$ S-methionine/cysteine, then chased for the indicated time. Metabolically labeled cultures  
35      were separated into both conditioned media and cells,

and detergent extracts were prepared from each, clarified, then immunoprecipitated with anti-OPG antibodies. The immunoprecipitates were resolved by SDS-PAGE, and exposed to film. Top left and right panels; samples analyzed under non-reducing conditions. Lower left and right panels; samples analyzed under reducing conditions. Top and bottom left panels; Cell extracts. Top and bottom right panels; Conditioned media extracts. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 17. Expression of OPG in the CTLL-2 cell line. Serum-free conditioned media from CTLL-2 cells and CHO-mu OPG [1-401] transfected cells was prepared, concentrated, then analyzed by non-reducing SDS-PAGE and western blotting. Left lane; CTLL-2 conditioned media. Right lane; CHO-muOPG conditioned media. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 18. Detection of OPG expression in serum samples and liver extracts obtained from control and OPG transgenic mice. Transgenic mice were constructed as described in Example 4. OPG expression was visualized after SDS-PAGE followed by Western blotting using anti-OPG antibodies.

Figure 19. Effects of huOPG [22-401]-Fc fusion protein on osteoclast formation in vitro. The osteoclast forming assay was performed as described in Example 11A in the absence (control) or presence of the indicated amounts of huOPG [22-401]-Fc fusion. Osteoclast formation was visualized by histochemical staining for tartrate acid phosphatase (TRAP). A. OPG added to 100 ng/ml. D. OPG added to 0.1 ng/ml. E. OPG added to 0.01 ng/ml. F. OPG added to 0.001 ng/ml. G. Control. No OPG added.

Figure 20. Decrease in osteoclast culture TRAP activity with increasing amounts of OPG. Indicated concentrations of huOPG [22-401]-Fc fusion protein were added to osteoclast forming assay and TRAP activity quantitated as described in Example 11A.

Figure 21. Effect of OPG on a terminal stage of osteoclast differentiation. huOPG [22-401]-Fc fusion was added to the osteoclast forming assay during the intermediate stage of osteoclast maturation (days 5-6; OPG-CTL) or during the terminal stage of osteoclast maturation (days 7-15; CTL-OPG). TRAP activity was quantitated and compared with the activity observed in the absence of OPG (CTL-CTL) in the presence of OPG throughout (OPG-OPG).

Figure 22. Effects of IL-1 $\beta$ , IL-1 $\alpha$  and OPG on blood ionized calcium in mice. Levels of blood ionized calcium were monitored after injection of IL-1 $\beta$  alone, IL-1 $\alpha$  alone, IL-1 $\beta$  plus muOPG [22-401]-Fc, IL-1 $\alpha$  plus MuOPG [22-401]-Fc, and muOPG [22-401]-Fc alone. Control mice received injections of phosphate buffered saline (PBS) only. IL-1 $\beta$  experiment shown in A; IL-1 $\alpha$  experiment shown in B.

Figure 23. Effects of OPG on calvarial osteoclasts in control and IL-1-treated mice. Histological methods for analyzing mice calvarial bone samples are described in Example 11B. Arrows indicate osteoclasts present in day 2-treated mice. Calvarial samples of mice receiving four PBS injections daily (A), one injection of IL-1 and three injections of PBS daily (B), one injection of PBS and three injections of OPG daily (C), one injection of IL-1 and three injections of OPG daily.

Figure 24. Radiographic analysis of bone accumulation in marrow cavity of normal mice. Mice were injected subcutaneously with saline (A) or muOPG [22-

401]-Fc fusion (5mg/kg/d) for 14 days (B) and bone density determined as described in Example 11C.

Figure 25. Histomorphometric analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 5 11C.

Figure 26. Histology analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 10 11C. A. Saline injection B. Injection of muOPG [22-401]-Fc fusion.

Figure 27. Activity of OPG administered to ovariectomized rats. In this two week experiment the trend to reduced bone density appears to be blocked by 15 OPG or other anti-resorptive therapies. DEXA measurements were taken at time of ovariectomy and at week 1 and week 2 of treatment. The results are expressed as % change from the initial bone density (Mean +/- SEM).

Figure 28. Bone density in the femoral metaphysis, measured by histomorphometric methods, tends to be lower in ovariectomized rats (OVX) than sham operated animals (SHAM) 17 days following ovariectomy. This effect was blocked by OPG-Fc, with OPG-Fc treated 25 ovariectomized rats (OVX+OPG) having significantly higher bone density than vehicle treated ovariectomized rats (OVX). (Mean +/- SEM).

Figure 29A through 29G. Sequence of OPG-Fc. DNA and encoded protein sequences are shown. Restriction sites for various nucleases are noted above the DNA sequence.

Figures 30A through 30D. Effects of OPG-Fc during the course of adjuvant arthritis I male Lewis rats. Paws from rats with adjuvant arthritis induced by 0.5mg 35 mycobacteria in oil were analyzed by DEXA for bone mineral density (BMD). Evaluation of BMD, a 29mm X 25mm

box was centered at the calcaneus (expt AdA-14 2/99, Amgen nb#22957 p47-49). \* compared to normal, # compared to vehicle P<0.05 Mann-Whitney U test.

Figures 31A and 31B. Combination treatment with  
5 OPG-Fc and sTNF-RI on Adjuvant Arthritis in Male Lewis  
Rats. Area under the curve (AUC) for measurement of paw  
swelling and BMD were measured as described above for  
Figure 33 and in the examples hereinafter.

Detailed Description of the InventionOPG proteins

The term "OPG protein" refers collectively to the novel member of the tumor necrosis factor receptor family described hereinafter, variants and truncations thereof that maintain OPG's activity in increasing bone density, and antibodies to OPG ligand that maintain OPG's activity in increasing bone density. An exemplary assay for measuring such activity is shown in figure 6 and the accompanying text. Exemplary OPG proteins are polypeptides comprising the consensus of the rat, mouse and human sequences (figure 9C), OPG-Fc fusions (figures 13, 29), or the rat, mouse or human OPG sequences (figures 2, 9).

OPG was identified as follows. A novel member of the tumor necrosis factor receptor (TNFR) superfamily was identified as an expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library . The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in Examples 1 and 6. The rat, mouse and human genes are shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), respectively. All three sequences showed strong similarity to the extracellular domains of TNFR family members. None of the full-length cDNA clones isolated encoded transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human gene spanning nucleotides 1200-1353 shown in Figure 9D was deposited in the Genebank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat, mRNA expression was detected in kidney, liver, placenta and

heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the same tissues along with lymph node, thymus, spleen and 5 appendix.

The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long 10 bones (femurs), vertebrae and flat bones (pelvis). Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and 15 resorption which has led to a marked accumulation of bone and cartilage. A decrease in the number of trabecular osteoclasts in the bones of OPG expressor animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent 20 bone resorption, a process mediated by osteoclasts. In view of the activity in transgenic expressors, the TNFR-related proteins described herein are termed OPGs.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse OPG in 293 cells and human OPG in E. coli is described 25 in Examples 7 and 8. Mouse OPG was produced as an Fc fusion which was purified by Protein A affinity chromatography. Also described in Example 7 is the expression of full-length and truncated human and mouse OPG polypeptides in CHO and 293 cells either as fusion 30 polypeptides to the Fc region of human IgG1 or as unfused polypeptides. The expression of full-length and truncated human and mouse OPGs in E. coli either as Fc fusion polypeptides or as unfused polypeptides is described in Example 8. Purification of recombinantly 35 produced mammalian and bacterial OPG is described in Example 10.

The biological activity of OPG was determined using an in vitro osteoclast maturation assay, an in vivo model of interleukin-1 (IL-1) induced hypercalcemia, and injection studies of bone density in 5 normal mice (see Example 11). The following OPG recombinant proteins produced in CHO or 293 cells demonstrated activity in the in E. coli osteoclast maturation assay: muOPG [22-185]-Fc, muOPG [22-194]-Fc, muOPG [22-401]Fc, muOPG [22-401], huOPG [22-201]-Fc, 10 huOPG [22-401]-Fc. muOPG [22-180]-Fc produced in CHO cells and huOPG met[32-401] produced in E. coli did not demonstrate activity in the in vitro assay.

OPG from several sources was produced as a dimer and to some extent as a higher multimer. Rat OPG [22- 15 401] produced in transgenic mice, muOPG [22-401] and huOPG [22-401] produced as a recombinant polypeptide in CHO cells, and OPG expressed as a naturally occurring product from a cytotoxic T cell line were predominantly dimers and trimers when analyzed on nonreducing SDS 20 gels (see Example 9). Truncated OPG polypeptides having deletions in the region of amino acids 186-401 (e.g., OPG [1-185] and OPG [1-194]) were predominantly monomeric suggesting that the region 186-401 may be involved in self-association of OPG polypeptides. 25 However, huOPG met[32-401] produced in E. coli was largely monomeric.

OPG may be important in regulating bone resorption. The protein appears to act as a soluble receptor of the TNF family and may prevent a receptor- 30 ligand interaction involved in the osteolytic pathway. One aspect of the regulation appears to be a reduction in the number of osteoclasts.

OPG proteins encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]- 35 Fc fusion, rat [1-180]-Fc fusion, mouse [1-401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180],

human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO:121 (rat), SEQ ID NO:123 (mouse) and SEQ ID NO:125 (human). Also  
5 encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of OPG; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of OPG, in  
10 particular deletion of the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain. In one embodiment, OPG has from 1 to about 216 amino acids  
15 deleted from the carboxy terminus. In another embodiment, OPG has from 1 to about 10 amino acids deleted from the mature amino terminus (wherein the mature amino terminus is at residue 22) and, optionally, has from 1 to about 216 amino acids deleted  
20 from the carboxy terminus.

Additional OPG proteins encompassed by the invention include the following: human [22-180]-Fc fusion, human [22-201]-Fc fusion, human [22-401]-Fc fusion, mouse [22-185]-Fc fusion, mouse [22-194]-Fc fusion. These polypeptides are produced in mammalian host cells, such as CHO or 293 cells, Additional OPG polypeptides encompassed by the invention which are expressed in prokaryotic host cells include the following: human met[22-401], Fc-human met[22-401] fusion (Fc region is fused at the amino terminus of the full-length OPG coding sequence as described in Example 8), human met[22-401]-Fc fusion (Fc region fused to the full-length OPG sequence), Fc-mouse met[22-401] fusion, mouse met[22-401]-Fc fusion, human met[27-401], human met[22-185], human met[22-189], human met[22-194], human met[22-194] (P25A), human met [22-194] (P26A),  
30  
35

human met[27-185], human met[27-189], human met[27-194], human met-arg-gly-ser-(his)<sub>6</sub> [22-401], human met-lys [22-401], human met-(lys)<sub>3</sub>-[22-401], human met[22-401]-Fc (P25A), human met[22-401] (P25A), human  
5 met[22-401] (P26A), human met[22-401] (P26D), mouse met[22-401], mouse met[27-401], mouse met[32-401], mouse met[27-180], mouse met[22-189], mouse met[22-194], mouse met[27-189], mouse met[27-194], mouse  
10 met-lys[22-401], mouse HEK[22-401] (A45T), mouse met-lys-(his)<sub>7</sub>[22-401], mouse met-lys[22-401]-(his)<sub>7</sub> and mouse met[27-401] (P33E, G36S, A45P). It is understood that the above OPG polypeptides produced in prokaryotic host cells have an amino-terminal methionine residue, if such a residue is not indicated. In specific  
15 examples, OPG-Fc fusion were produced using a 227 amino acid region of human IgG1- $\gamma$ 1 was used having the sequence as shown in Ellison *et al.* (1982) *Nuc. Acids Res.* 10: 4071-9. However, variants of the Fc region of human IgG may also be used.

20 Analysis of the biological activity of carboxy-terminal OPG truncations fused to the human IgG1 Fc region indicates a portion of OPG of about 164 amino acids which is required for activity. This region encompasses amino acids 22-185, preferably those in  
25 Figure 9C-9D (SEQ ID NO:125), and comprises four cysteine-rich domains characteristic of the cysteine-rich domains of TNFR extracellular domains. Proteins comprising this 164 amino acid sequence are within the meaning of "OPG protein" in this invention.

30 OPG proteins of the invention also may be isolated and purified from other polypeptides present in tissues, cell lines and transformed host cells expressing OPG, or purified from components in cell cultures containing the secreted protein. In one embodiment, the polypeptide is free from association  
35

with other human proteins, such as the expression product of a bacterial host cell.

A method for the purification of OPG from natural sources and from transfected host cells is also included. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG antibody or biotin-streptavidin affinity complex and the like.

#### IL-1 inhibitors

One of the most potent inflammatory cytokines yet discovered is interleukin-1 (IL-1). IL-1 is thought to be a key mediator in many diseases and medical conditions. It is manufactured (though not exclusively) by cells of the macrophage/monocyte lineage and may be produced in two forms: IL-1 alpha (IL-1 $\alpha$ ) and IL-1 beta (IL-1 $\beta$ ).

A disease or medical condition is considered to be an "interleukin-1 mediated disease" if the spontaneous or experimental disease or medical condition is associated with elevated levels of IL-1 in bodily fluids or tissue or if cells or tissues taken from the body produce elevated levels of IL-1 in culture. In many cases, such interleukin-1 mediated diseases are also recognized by the following additional two conditions: (1) pathological findings associated with the disease or medical condition can be mimicked experimentally in animals by administration of IL-1 or upregulation of expression of IL-1; and (2) a pathology induced in experimental animal models of the disease or medical condition can be inhibited or abolished by treatment with agents that inhibit the action of IL-1. In most interleukin-1 mediated diseases at least two of

the three conditions are met, and in many interleukin-1 mediated diseases all three conditions are met.

A non-exclusive list of acute and chronic interleukin-1 (IL-1)-mediated diseases includes but is  
5 not limited to the following:

acute pancreatitis;  
ALS;  
Alzheimer's disease;  
cachexia/anorexia, including AIDS-induced  
10 cachexia;  
asthma and other pulmonary diseases;  
atherosclerosis;  
autoimmune vasculitis;  
chronic fatigue syndrome;  
15 Clostridium associated illnesses, including  
Clostridium-associated diarrhea;  
coronary conditions and indications,  
including congestive heart failure, coronary  
restenosis, myocardial infarction, myocardial  
20 dysfunction (e.g., related to sepsis), and coronary  
artery bypass graft;  
cancer, such as multiple myeloma and  
myelogenous (e.g., AML and CML) and other leukemias, as  
well as tumor metastasis;  
25 diabetes (e.g., insulin diabetes);  
endometriosis;  
fever;  
fibromyalgia;  
glomerulonephritis;  
30 graft versus host disease/transplant  
rejection;  
hemorrhagic shock;  
hyperalgesia;  
inflammatory bowel disease;

inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis;

inflammatory eye disease, as may be associated with, for example, corneal transplant; ischemia, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration);

Kawasaki's disease;

learning impairment;

lung diseases (e.g., ARDS);

multiple sclerosis;

myopathies (e.g., muscle protein metabolism, esp. in sepsis);

neurotoxicity (e.g., as induced by HIV);

osteoporosis;

pain, including cancer-related pain;

Parkinson's disease;

periodontal disease;

pre-term labor;

psoriasis;

reperfusion injury;

septic shock;

side effects from radiation therapy;

temporal mandibular joint disease;

sleep disturbance;

uveitis;

or an inflammatory condition resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes.

Interleukin-1 inhibitors may be from any protein capable of specifically preventing activation of cellular receptors to IL-1, which may result from any number of mechanisms. Such mechanisms include downregulating IL-1 production, binding free IL-1,

interfering with IL-1 binding to its receptor,  
interfering with formation of the IL-1 receptor complex  
(i.e., association of IL-1 receptor with IL-1 receptor  
accessory protein), or interfering with modulation of  
5 IL-1 signaling after binding to its receptor. Classes  
of interleukin-1 inhibitors include:

interleukin-1 receptor antagonists such as IL-1ra,  
as described below;  
anti-IL-1 receptor monoclonal antibodies (e.g., EP  
10 623674), the disclosure of which is hereby incorporated  
by reference;

IL-1 binding proteins such as soluble IL-1  
receptors (e.g., U. S. Pat. No. 5,492,888, U. S. Pat.  
No. 5,488,032, and U. S. Pat. No. 5,464,937, U. S. Pat.  
15 No. 5,319,071, and U.S. Pat. No. 5,180,812, the  
disclosures of which are hereby incorporated by  
reference);

anti-IL-1 monoclonal antibodies (e.g., WO 9501997,  
WO 9402627, WO 9006371, U.S.Pat. No. 4,935,343, EP  
20 364778, EP 267611 and EP 220063, the disclosures of  
which are hereby incorporated by reference);

IL-1 receptor accessory proteins and antibodies  
thereto (e.g., WO 96/23067 and WO 99/37773, the  
disclosure of which is hereby incorporated by  
25 reference);

inhibitors of interleukin-1 beta converting enzyme  
(ICE) or caspase I (e.g., WO 99/46248, WO 99/47545, and  
WO 99/47154, the disclosures of which are hereby  
incorporated by reference), which can be used to  
30 inhibit IL-1 beta production and secretion;

interleukin-1beta protease inhibitors;  
and other compounds and proteins which block *in  
vivo* synthesis or extracellular release of IL-1.

Exemplary IL-1 inhibitors are disclosed in the  
35 following references:

US Pat. Nos. 5,747,444; 5,359,032; 5,608,035;  
5,843,905; 5,359,032; 5,866,576; 5,869,660; 5,869,315;  
5,872,095; 5,955,480; 5,965,564;

International (WO) patent applications 98/21957,  
5 96/09323, 91/17184, 96/40907, 98/32733, 98/42325,  
98/44940, 98/47892, 98/56377, 99/03837, 99/06426,  
99/06042, 91/17249, 98/32733, 98/17661, 97/08174,  
95/34326, 99/36426, 99/36415.

European (EP) patent applications 534978 and  
10 894795.

French patent application FR 2762514.

The disclosures of all of the aforementioned references  
are hereby incorporated by reference.

For purposes of the present invention, IL-1ra and  
15 variants and derivatives thereof as discussed  
hereinafter are collectively termed "IL-1ra  
protein(s)". The molecules described in the above  
references and the variants and derivatives thereof  
discussed hereinafter are collectively termed "IL-1  
20 inhibitors."

Interleukin-1 receptor antagonist (IL-1ra) is a  
human protein that acts as a natural inhibitor of  
interleukin-1 and which is a member of the IL-1 family  
member which includes IL-1 $\alpha$  and IL-1 $\beta$ . Preferred  
25 receptor antagonists (including IL-1ra and variants and  
derivatives thereof), as well as methods of making and  
using thereof, are described in U.S. Patent No.  
5,075,222; WO 91/08285; WO 91/17184; AU 9173636;  
WO 92/16221; WO93/21946; WO 94/06457; WO 94/21275;  
30 FR 2706772; WO 94/21235; DE 4219626, WO 94/20517; WO  
96/22793; WO 97/28828; and WO 99/36541, the disclosures  
of which are incorporated herein by reference. The  
proteins include glycosylated as well as non-  
glycosylated IL-1 receptor antagonists.

Specifically, three useful forms of IL-1ra and variants thereof are disclosed and described in the 5,075,222 patent. The first of these, called "IL-1i" in the '222 patent, is characterized as a 22-23 kD molecule on SDS-PAGE with an approximate isoelectric point of 4.8, eluting from a Mono Q FPLC column at around 52 mM NaCl in Tris buffer, pH 7.6. The second, IL-1ra $\beta$ , is characterized as a 22-23 kD protein, eluting from a Mono Q column at 48 mM NaCl. Both IL-1ra $\alpha$  and IL-1ra $\beta$  are glycosylated. The third, IL-1rax, is characterized as a 20 kD protein, eluting from a Mono Q column at 48 mM NaCl, and is non-glycosylated. 5,075,222 patent also discloses methods for isolating the genes responsible for coding the inhibitors, cloning the gene in suitable vectors and cell types, and expressing the gene to produce the inhibitors.

Those skilled in the art understand that many combinations of deletions, insertions and substitutions (individually or collectively "variant(s)") can be made within the amino acid sequences of IL-1ra, provided that the resulting molecule is biologically active (e.g., possesses the ability to inhibit IL-1). See "Variants of Proteins" hereinafter.

#### TNF- $\alpha$ inhibitors

Many diseases and medical conditions are mediated by TNF and are usually categorized as inflammatory conditions. A "TNF-mediated disease" is a spontaneous or experimental disease or medical condition is associated with elevated levels of TNF in bodily fluids or tissue or if cells or tissues taken from the body produce elevated levels of TNF in culture. In many cases, such TNF-mediated diseases may also be recognized by (1) pathological findings associated with the disease or medical condition can be mimicked experimentally in animals by the administration or

upregulation of expression of TNF or (2) a pathology induced in experimental animal models of the disease or medical condition can be inhibited or abolished by treatment with agents that inhibit the action of TNF. A 5 non-exclusive list of acute and chronic TNF-mediated diseases includes but is not limited to the following:

cachexia/anorexia;

cancer (e.g., leukemias);

chronic fatigue syndrome;

10 coronary conditions and indications, including congestive heart failure, coronary restenosis, myocardial infarction, myocardial dysfunction (e.g., related to sepsis), and coronary artery bypass graft;

depression;

15 diabetes, including juvenile onset Type 1, diabetes mellitus, and insulin resistance (e.g., as associated with obesity);

endometriosis, endometritis, and related conditions;

20 fibromyalgia or analgesia;

graft versus host rejection;

hyperalgesia;

inflammatory bowel diseases, including Crohn's disease and Clostridium difficile-associated diarrhea;

25 ischemia, including cerebral ischemia (brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration);

lung diseases (e.g., adult respiratory distress syndrome, asthma, and pulmonary fibrosis);

30 multiple sclerosis;

neuroinflammatory diseases;

ocular diseases and conditions, including corneal transplant, ocular degeneration and uveitis;

pain, including cancer-related pain;

35 pancreatitis;

periodontal diseases;

Pityriasis rubra pilaris (PRP);  
prostatitis (bacterial or non-bacterial) and  
related conditions;  
psoriasis and related conditions;  
5 pulmonary fibrosis;  
reperfusion injury;  
rheumatic diseases, including rheumatoid  
arthritis, osteoarthritis, juvenile (rheumatoid)  
arthritis, seronegative polyarthritis, ankylosing  
10 spondylitis, Reiter's syndrome and reactive arthritis,  
Still's disease, psoriatic arthritis, enteropathic  
arthritis, polymyositis, dermatomyositis, scleroderma,  
systemic sclerosis, vasculitis (e.g., Kawasaki's  
disease), cerebral vasculitis, Lyme disease,  
15 staphylococcal-induced ("septic") arthritis, Sjögren's  
syndrome, rheumatic fever, polychondritis and  
polymyalgia rheumatica and giant cell arteritis);  
septic shock;  
side effects from radiation therapy;  
20 systemic lupus erythematosus (SLE);  
temporal mandibular joint disease;  
thyroiditis;  
tissue transplantation or an inflammatory  
condition resulting from strain, sprain, cartilage  
25 damage, trauma, orthopedic surgery, infection (e.g.,  
HIV, Clostridium difficile and related species) or  
other disease process.

TNF- $\alpha$  inhibitors may act by downregulating or  
inhibiting TNF production, binding free TNF,  
30 interfering with TNF binding to its receptor, or  
interfering with modulation of TNF signaling after  
binding to its receptor. The term "TNF- $\alpha$  inhibitor"  
thus includes solubilized TNF receptors, antibodies to  
TNF, antibodies to TNF receptor, inhibitors of TNF- $\alpha$

converting enzyme (TACE), and other molecules that affect TNF activity.

TNF- $\alpha$  inhibitors of various kinds are disclosed in the art, including the following references:

- 5 European patent applications 308 378; 422 339; 393  
438; 398 327; 412 486; 418 014, 417 563, 433 900; 464  
533; 512 528; 526 905; 568 928; EP 607 776 (use of  
leflunomide for inhibition of TNF- $\alpha$ ); 663 210; 542 795;  
818 439; 664 128; 542 795; 741 707; 874 819 ; 882 714;  
10 880 970; 648 783; 731 791; 895 988; 550 376; 882 714;  
853 083; 550 376; 943 616; 939 121; 614 984 ; 853 083  
U.S. Patent Nos. 5,136,021; 5,929,117; 5,948,638;  
5,807,862; 5,695,953; 5,834,435; 5,817,822; 5830742;  
5,834,435; 5,851,556; 5,853,977; 5,359,037; 5,512,544;  
15 5,695,953; 5,811,261; 5,633,145; 5,863,926; 5,866,616;  
5,641,673; 5,869,677; 5,869,511; 5,872,146; 5,854,003;  
5,856,161; 5,877,222; 5,877,200; 5,877,151; 5,886,010;  
5,869,660; 5,859,207; 5,891,883; 5,877,180; 5,955,480;  
5,955,476; 5,955,435; 5,994,351; 5,990,119; 5,952,320;  
20 5,962,481;  
International (WO) patent applications 90/13575,  
91/03553, 92/01002, 92/13095, 92/16221, 93/07863,  
93/21946, 93/19777, 95/34326, 96/28546, 98/27298,  
98/30541, 96/38150, 96/38150, 97/18207, 97/15561,  
25 97/12902, 96/25861, 96/12735, 96/11209, 98/39326,  
98/39316, 98/38859, 98/39315, 98/42659, 98/39329,  
98/43959, 98/45268, 98/47863, 96/33172, 96/20926,  
97/37974, 97/37973, 97/47599, 96/35711, 98/51665,  
98/43946, 95/04045, 98/56377, 97/12244, 99/00364,  
30 99/00363, 98/57936, 99/01449, 99/01139, 98/56788,  
98/56756, 98/53842, 98/52948, 98/52937, 99/02510,  
97/43250, 99/06410, 99/06042, 99/09022, 99/08688,  
99/07679, 99/09965, 99/07704, 99/06041, 99/37818,  
99/37625, 97/11668, 99/50238, 99/47672, 99/48491;

Japanese (JP) patent applications 10147531, 10231285, 10259140, and 10130149, 10316570, 11001481, and 127,800/1991;

5 German (DE) application 19731521;  
British (GB) applications 2 218 101, 2 326 881, 2  
246 569.

The disclosures of all of the aforementioned references are hereby incorporated by reference.

For purposes of this invention, the molecules  
10 disclosed in these references and the sTNFRs and variants and derivatives of the sTNFRs and the molecules disclosed in the references (see below) are collectively termed "TNF- $\alpha$  inhibitors."

For example, EP 393 438 and EP 422 339 teach the  
15 amino acid and nucleic acid sequences of a soluble TNF receptor type I (also known as sTNFR-I or 30kDa TNF inhibitor) and a soluble TNF receptor type II (also known as sTNFR-II or 40kDa TNF inhibitor), collectively termed "sTNFRs", as well as modified forms thereof  
20 (e.g., fragments, functional derivatives and variants). EP 393 438 and EP 422 339 also disclose methods for isolating the genes responsible for coding the inhibitors, cloning the gene in suitable vectors and cell types, and expressing the gene to produce the  
25 inhibitors.

sTNFR-I and sTNFR-II are members of the nerve growth factor/TNF receptor superfamily of receptors which includes the nerve growth factor receptor (NGF), the B cell antigen CD40, 4-1BB, the rat T-cell antigen MRC OX40, the fas antigen, and the CD27 and CD30 antigens (Smith *et al.* (1990), *Science*, 248:1019-1023). The most conserved feature amongst this group of cell surface receptors is the cysteine-rich extracellular ligand binding domain, which can be divided into four  
30 repeating motifs of about forty amino acids and which  
35

contains 4-6 cysteine residues at positions which are well conserved (Smith *et al.* (1990), *supra*).

EP 393 438 teaches a 40kDa TNF inhibitor Δ51 and a 40kDa TNF inhibitor Δ53, which are truncated versions 5 of the full-length recombinant 40kDa TNF inhibitor protein wherein 51 or 53 amino acid residues, respectively, at the carboxyl terminus of the mature protein are removed.

PCT Application No. PCT/US97/12244 teaches 10 truncated forms of sTNFR-I and sTNFR-II which do not contain the fourth domain (amino acid residues Thr<sup>127</sup>-Asn<sup>161</sup> of sTNFR-I and amino acid residues Pro<sup>141</sup>-Thr<sup>179</sup> of sTNFR-II); a portion of the third domain (amino acid residues Asn<sup>111</sup>-Cys<sup>126</sup> of sTNFR-I and amino acid 15 residues Pro<sup>123</sup>-Lys<sup>140</sup> of sTNFR-II); and, optionally, which do not contain a portion of the first domain (amino acid residues Asp<sup>1</sup>-Cys<sup>19</sup> of sTNFR-I and amino acid residues Leu<sup>1</sup>-Cys<sup>32</sup> of sTNFR-II). The truncated sTNFRs of the present invention include the proteins 20 represented by the formula R<sub>1</sub>-[Cys<sup>19</sup>-Cys<sup>103</sup>]-R<sub>2</sub> and R<sub>4</sub>-[Cys<sup>32</sup>-Cys<sup>115</sup>]-R<sub>5</sub>. These proteins are truncated forms of sTNFR-I and sTNFR-II, respectively.

By "R<sub>1</sub>-[Cys<sup>19</sup>-Cys<sup>103</sup>]-R<sub>2</sub>" is meant one or more 25 proteins wherein [Cys<sup>19</sup>-Cys<sup>103</sup>] represents residues 19 through 103 of sTNFR-I, the amino acid residue numbering scheme of which is provided in Figure 1 to facilitate the comparison; wherein R<sub>1</sub> represents a methionylated or nonmethionylated amine group of Cys<sup>19</sup> or of amino-terminus amino acid residue(s) selected 30 from any one of Cys<sup>18</sup> to Asp<sup>1</sup> and wherein R<sub>2</sub> represents a carboxy group of Cys<sup>103</sup> or of carboxy-terminal amino acid residues selected from any one of Phe<sup>104</sup> to Leu<sup>110</sup>.

Exemplary truncated sTNFR-I of the present invention include the following molecules (collectively

termed 2.6D sTNFR-I): NH<sub>2</sub>-[Asp<sup>1</sup>-Cys<sup>105</sup>]-COOH (also referred to as sTNFR-I 2.6D/C105); NH<sub>2</sub>-[Asp<sup>1</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.6D/C106); NH<sub>2</sub>-[Asp<sup>1</sup>-Asn<sup>105</sup>]-COOH (also referred to as sTNFR-I 5 2.6D/N105); NH<sub>2</sub>-[Tyr<sup>9</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.3D/d8); NH<sub>2</sub>-[Cys<sup>19</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.3D/d18); and NH<sub>2</sub>-[Ser<sup>16</sup>-Leu<sup>108</sup>]-COOH (also referred to as sTNFR-I 2.3D/d15), either methionylated or nonmethionylated, and variants and 10 derivatives thereof.

By "R<sub>3</sub>-[Cys<sup>32</sup>-Cys<sup>115</sup>]-R<sub>4</sub>" is meant one or more proteins wherein [Cys<sup>32</sup>-Cys<sup>115</sup>] represents residues Cys<sup>32</sup> through Cys<sup>115</sup> of sTNFR-II, the amino acid residue 15 numbering scheme of which is provided in Figure 2 to facilitate the comparison; wherein R<sub>3</sub> represents a methionylated or nonmethionylated amine group of Cys<sup>32</sup> or of amino-terminus amino acid residue(s) selected from any one of Cys<sup>31</sup> to Leu<sup>1</sup> and wherein R<sub>4</sub> represents a carboxy group of Cys<sup>115</sup> or of carboxy-terminal amino. 20 acid residue(s) selected from any one of Ala<sup>116</sup> to Arg<sup>122</sup>.

Serine Protease Inhibitors

Endogenous proteolytic enzymes degrade invading organisms, antigen-antibody complexes, and certain tissue proteins that are no longer necessary or useful.

5 Infective agents may introduce additional proteolytic enzymes into the organism. Protease inhibitors regulate both endogenous and invading proteolytic enzymes.

A large number of naturally occurring protease inhibitors serve to control the endogenous proteases by

10 limiting their reactions locally and temporally. In addition, the protease inhibitors may inhibit proteases introduced into the body by infective agents. Tissues that are particularly prone to proteolytic attack and infection, e.g. those of the respiratory tract, are

15 rich in protease inhibitors.

Protease inhibitors comprise approximately 10% of the human plasma proteins. At least eight inhibitors have been isolated from this source and characterized in the literature. These include alpha 2-macroglobulin

20 (alpha 2M), alpha 1-protease inhibitor (alpha 1PI), alpha 1-antichymotrypsin (alpha 1AChy), alpha 1-anticollagenase (alpha 1AC), and inter-alpha-trypsin inhibitor (I-alpha-I).

A disturbance of the protease/protease inhibitor balance can lead to protease-mediated tissue destruction, including emphysema, arthritis, glomerulonephritis, periodontitis, muscular dystrophy, tumor invasion and various other pathological conditions. In certain situations, e.g. severe

25 pathological processes such as sepsis or acute leukemia, the amount of free proteolytic enzymes present increases due to the release of enzyme from the secretory cells. In addition, or separately in other situations, a diminished regulating inhibitor capacity

of the organism may also cause alterations in the protease/protease inhibitor balance. An example of such a diminished regulating inhibitor capacity is an alpha 1-protease inhibitor deficiency, which is highly  
5 correlated with the development of pulmonary emphysema.

In organisms where such aberrant conditions are present, serious damage to the organism can occur unless measures can be taken to control the proteolytic enzymes. Therefore, protease inhibitors have been  
10 sought which are capable of being administered to an organism to control the proteolytic enzymes.

One protease that is of particular pharmacological interest is leukocyte elastase. Leukocyte elastase, when released extracellularly, degrades connective  
15 tissue and other valuable proteins. While it is necessary for a normally functioning organism to degrade a certain amount of connective tissue and other proteins, the presence of an excessive amount of leukocyte elastase has been associated with various  
20 pathological states, such as emphysema and rheumatoid arthritis. To counteract the effects of leukocyte elastase when it is present in amounts greater than normal, a protease inhibitor has been sought which is specific for leukocyte elastase. Such a protease  
25 inhibitor would be especially useful if it were capable of being isolated or prepared in a purified form and in sufficient quantities to be pharmaceutically useful.

In the past, at least two leukocyte elastase inhibitors have been identified in the literature. One  
30 protein, described in Schiessler *et al.*, "Acid-Stable Inhibitors of Granulocyte Neutral Proteases in Human Mucous Secretions: Biochemistry and Possible Biological Function", in Neutral Proteases of Human

Polymorphonuclear Leucocytes, Havemann *et al.* (eds), Urban and Schwarzenberg, Inc. (1978), was isolated from human seminal plasma and sputum and was characterized as being approximately 11 Kda in size with tyrosine as 5 the N-terminal amino acid. The literature reports of this protein have only furnished a partial amino acid sequence, but even this partial sequence indicates that this protein varies substantially from the proteins of the present invention. The reports of the sequence of 10 this protein, in combination with the complete amino acid sequence data for proteins of the present invention, indicate to the present inventors that the product sequenced by Schiessler *et al.* may have been a degraded protein which was not a single-polypeptide 15 chain.

A second protein, isolated in one instance from human plasma, has been named alpha 1-protease inhibitor. Work on this protein has been summarized in a review by Travis and Salvesen, Ann. Rev. Biochem. 52: 20 655-709 (1983). The reports of the amino acid sequence of this protein indicate that it too differs substantially from the proteins of the present invention.

Trypsin is another protease of particular interest 25 from a pharmacological standpoint. Trypsin is known to initiate degradation of certain soft organ tissue, such as pancreatic tissue, during a variety of acute conditions, such as pancreatitis. A variety of efforts have been directed toward the treatment of these 30 conditions, without marked success, through the use of proteins which it was hoped would inhibit the action of trypsin. Illustrative of such efforts are attempts to use exogenous bovine trypsin inhibitors in treatment of

human pancreatitis. While such techniques have been attempted in Europe, they have not been approved as effective by the U.S. Food and Drug Administration.

Thus, there is a need for a protease inhibitor  
5 effective in neutralizing excess trypsin in a variety  
of acute and chronic conditions. As was the case with  
the leukocyte elastase inhibitor discussed above, a  
trypsin inhibitor would be particularly useful if it  
could be isolated and prepared in a purified form and  
10 in sufficient quantities to be pharmaceutically useful.

Cathepsin G is another protease present in large  
quantities in leukocytes. Cathepsin G is known to be  
capable of degrading in vitro a variety of valuable  
proteins, including those of the complement pathway  
15 Pancreatic elastase is another protease which may have  
a role in pancreatitis. Thus, inhibitors for these  
proteases are also of pharmaceutical value.

Leukocyte elastase, trypsin, cathepsin G and  
pancreatic elastase are examples of a class of  
20 proteases known as serine proteases, which have  
elements of common structure and mechanism. Their  
activity against different substrates and their  
sensitivity to different inhibitors are believed to  
result from changes in only a few amino acid residues.  
25 By analogy, it is possible to conceive of a class of  
serine protease inhibitors, also having common elements  
of structure and mechanism, in which changes in a  
relatively few amino acids will result in inhibition of  
different proteases, and that at least one member of  
30 this class will inhibit every serine protease of the  
former class.

A particularly preferred serine protease inhibitor  
is secretory leukocyte protease inhibitor (SLPI) and

fragments and analogues thereof. Also preferred are anti-leukoprotease (ALP), mucous protease inhibitor (MPI), human seminal plasma inhibitor-I (HUSI-I), bronchial mucus inhibitor (BMI), cervical mucus inhibitor (CUSI). These molecules are especially well-suited for use in conditions leading to bone loss because they are preferentially directed to the cartilage. Exemplary serine protease inhibitors are described in the following, each of which is hereby incorporated by reference: U. S. Pat. No. 4,760,130, issued July 26, 1988; U. S. Pat. No. 5,900,400, issued May 4, 1999, which discloses preferred SLPI analogues; and U. S. Pat. No. 5,633,227, issued May 27, 1997, which discloses preferred SLPI fragments. The molecules disclosed in the foregoing references as well as any variants or analogues thereof as described hereinafter are collectively termed "serine protease inhibitors."

#### IL-18 Inhibitors

IL-18 is a pro-inflammatory cytokine of somewhat recent discovery. IL-18 was found to induce interferon- $\gamma$  and was originally named interferon gamma inducing factor (IGIF). IL-1 upregulates IL-18 production, and IL-18 induces production of a number of proinflammatory cytokines, including IL-6 and MMP-1. Dinarello *et al.* (1998), *J. Leukocyte Biol.* 63: 658-64. Caspase I is also critical for IL-18 production. The art also suggested that TNF- $\alpha$  regulates IL-18 production, and it was found that simultaneous inhibition of TNF- $\alpha$  and IL-18 protected against liver toxicity. Faggioni *et al.* (2000), *PNAS* 97: 2367-72.

IL-18 acts *in vivo* through a receptor system reminiscent of the IL-1 system. IL-18 interacts with a cell surface receptor (IL-18R), which interacts with an accessory protein (IL-18R $\alpha$ ). IL-18-mediated signaling proceeds upon formation of the complex of IL-18, IL-

18R, and IL-18RAcP. A natural inhibitor for IL-18 is IL-18bp. Although it bears insignificant sequence homology with IL-18R, IL-18bp's act as a "decoy receptors" by binding to IL-18 molecules and preventing 5 interaction with IL-18 and subsequent IL-18-mediated signaling.

The present invention concerns methods of treatment using IL-18 inhibitors in combination with the other classes of molecules described herein. Such 10 combination therapy is useful for treating inflammation and autoimmune diseases generally, as well as IL-1 mediated diseases and TNF-mediated diseases as defined hereinabove. In particular, combination therapy using IL-18 inhibitors is useful for treating arthritis 15 (particularly rheumatoid arthritis), systemic lupus erythematosus (SLE), graft versus host disease (GvHD), hepatitis and sepsis.

A number of classes of IL-18 inhibitors are known in the art, and all are useful in the present 20 invention. Suitable IL-18 inhibitors include antibodies binding to IL-18; antibodies binding to IL-18R; antibodies binding to IL-18RAcP; IL-18bp; IL-18R fragments (e.g., a solubilized extracellular domain of the IL-18 receptor), peptides binding to IL-18 and 25 preventing its interaction with IL-18R; peptides binding to IL-18R and preventing its interaction with IL-18 or with IL-18RAcP; peptides binding to IL-18RAcP and preventing its interaction with IL-18R; and small molecules preventing IL-18 production or interaction 30 between any of IL-18, IL-18R, and IL-18RAcP. Any of the foregoing, with the exception of small molecules, may be linked to half-life extending vehicles known in the art. Such vehicles include the Fc domain, polyethylene glycol, and dextran. These vehicles are reviewed in a 35 patent application entitled, "Modified Peptides as Therapeutic Agents," U.S. Ser. No. 09/428,082, PCT

appl. no. WO 99/25044, which is hereby incorporated by reference in its entirety.

Useful IL-18 inhibitors are described in the following references, which are hereby incorporated by reference: US Pat. No. 5,912,324, issued July 14, 1994; EP 0 962 531, published Dec. 8, 1999; EP 712 931, published Nov. 15, 1994; US Pat. No. 5,914,253, issued July 14, 1994; WO 97/24441, published July 10, 1997; US Pat. No. 6,060,283, issued May 9, 2000; EP 850 952, published Dec. 26, 1996; EP 864 585, published Sep. 16, 1998; WO 98/41232, published Sep. 24, 1998; US Pat. No. 6,054,487, issued April 25, 2000; WO 99/09063, published Aug 14, 1997; WO 99/22760, published Nov. 3, 1997; WO 99/37772, published Jan. 23, 1998; WO 15 99/37773, published March 20, 1998; EP 0 974 600, published Jan. 26, 2000; WO 00/12555, published Mar. 9, 2000; Japanese patent application JP 111,399/94, published Oct. 31, 1997; Israel patent application IL 121554 A0, published Feb. 8, 1998.

20 Variants of proteins

Those skilled in the art will understand that one may make many molecules derived in sequence from the aforementioned molecules in which amino acids have been deleted ("deletion variants"), inserted ("addition variants"), or substituted ("substitution variants"). Molecules having such substitutions, additions, deletions, or any combination thereof are termed individually or collectively "variant(s)"). Such variants should, however, maintain at some level (including a reduced level) the relevant activity of the unmodified or "parent" molecule (e.g., an sTNFR variant possesses the ability to bind TNF). Hereinafter, "parent molecule" refers to an unmodified molecule or a variant molecule lacking the particular variation under discussion; for example, when

discussing substitution below, the parent molecule may be a deletion variant.

Variants may be rapidly screened to assess their physical properties. It will be appreciated that such 5 variant(s) will demonstrate similar properties to the unmodified molecule, but not necessarily all of the same properties and not necessarily to the same degree as the corresponding parent molecule.

There are two principal variables in the 10 construction of amino acid sequence variant(s): the location of the mutation site and the nature of the mutation. In designing variant(s), the location of each mutation site and the nature of each mutation will depend on the biochemical characteristic(s) to be 15 modified. Each mutation site can be modified individually or in series, e.g., by (1) deleting the target amino acid residue, (2) inserting one or more amino acid residues adjacent to the located site or (3) substituting first with conservative amino acid 20 choices and, depending upon the results achieved, then with more radical selections.

Amino acid sequence deletions generally range from about 1 to 30 amino acid residues, preferably from about 1 to 20 amino acid residues, more preferably from 25 about 1 to 10 amino acid residues and most preferably from about 1 to 5 contiguous residues. Amino-terminal, carboxy-terminal and internal intrasequence deletions are contemplated. Deletions within the amino acid sequences of OPG or the STNFRs may be made, for 30 example, in regions of low homology with the sequences of other members of the NGF/TNF receptor family. In the case of IL-1ra, deletions may be made in regions of low homology in the IL-1 family (which comprises IL-1  $\alpha$ , IL-1  $\beta$ , and IL-1ra). Deletions in areas of substantial 35 homology with other members of the family will be more

likely to significantly modify the biological activity. Specifically, the sequence similarity among NGF/TNF receptor family members is particularly high in the region corresponding to the first two disulfide loops  
5 of domain 1, the whole of domain 2, and the first disulfide loop of domain 3 (Banner *et al.* (1993), *Cell*, 73:431-445). The number of total deletions and/or consecutive deletions preferably will be selected so as to preserve the tertiary structure in the affected  
10 domain, e.g., cysteine crosslinking.

An amino acid sequence addition may include insertions of an amino- and/or carboxyl-terminal fusion ranging in length from one residue to one hundred or more residues, as well as internal intrasequence  
15 insertions of single or multiple amino acid residues. Internal additions may range generally from about 1 to 20 amino acid residues, preferably from about 1 to 10 amino acid residues, more preferably from about 1 to 5 amino acid residues, and most preferably from about 1  
20 to 3 amino acid residues. Additions within the amino acid sequences of OPG or the sTNFRs may be made in regions of low homology with the sequences of other members of the NGF/TNF receptor family. Additions within the amino acid sequence of OPG or the sTNFRs in  
25 areas of substantial homology with the sequences of other members of the NGF/TNF receptor family will be more likely to significantly modify the biological activity. Additions preferably include amino acid sequences derived from the sequences of the NGF/TNF  
30 receptor family members.

An amino-terminus addition is contemplated to include the addition of a methionine (for example, as an artifact of the direct expression in bacterial recombinant cell culture). A further example of an  
35 amino-terminal addition includes the fusion of a signal sequence to the amino-terminus of a mature molecule in

order to facilitate its secretion from recombinant host cells. Such signal sequences generally will be obtained from and thus be homologous to the intended host cell species. For prokaryotic host cells that do not recognize and process the native signal sequence of the mature molecule, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase or heat-stable enterotoxin II leader sequences. For expression in yeast cells the signal sequence may be selected, for example, from the group of the yeast invertase, alpha factor or acid phosphatase leader sequences. For mammalian cell expression, the native signal sequences (see, e.g., EP 15 393 438 and EP 422 339 for sTNFRs) are satisfactory, although other mammalian signal sequences may be suitable; for example, sequences derived from other NGF/TNF receptor family members.

An example of an amino- or a carboxy-terminus addition includes chimeric proteins comprising the amino-terminal or carboxy-terminal fusion of the parent molecules with all or part of the constant domain of the heavy or light chain of human immunoglobulin (individually or collectively, ("Fc variant(s)"). Such chimeric polypeptides are preferred wherein the immunoglobulin portion of each comprises all of the domains except the first domain of the constant region of the heavy chain of human immunoglobulin such as IgG (e.g., IgG1 or IgG3), IgA, IgM or IgE. A skilled artisan will appreciate that any amino acid of the immunoglobulin portion can be deleted or substituted with one or more amino acids, or one or more amino acids can be added as long as the parent molecule still maintains some level of its relevant activity and the immunoglobulin portion shows one or more of its characteristic properties.

Another group of variant(s) is amino acid substitution variant(s). These are variant(s) wherein at least one amino acid residue in a parent molecule is removed and a different residue inserted in its place.

5 Substitution variant(s) include allelic variant(s) which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. One skilled in the art can use any information known about

10 the binding or active site of the polypeptide in the selection of possible mutation sites.

One method for identifying amino acid residues or regions for mutagenesis of a protein is called "alanine scanning mutagenesis", as described by Cunningham and Wells (1989), Science, 244:1081-1085, the disclosure of which is hereby incorporated by reference. In this method, an amino acid residue or group of target residues is identified (e.g., charged residues such as Arg, Asp, His, Lys and Glu) and replaced by a neutral or negatively-charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains/residues demonstrating functional sensitivity to the substitutions are then refined by introducing additional or alternate residues at the sites of substitution. Thus, the site for introducing an amino acid sequence modification is predetermined. To optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted and the variant(s) may be screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites in which particular amino acid residues within a parent molecule are substantially different from other species or other

family members in terms of side-chain bulk, charge and/or hydrophobicity. Other sites of interest include those in which particular residues of a parent molecule are identical among other species or other family 5 members, as such positions are generally important for the biological activity of a protein.

Other sites of interest include those in which particular residues are similar or identical with proteins with similar structure or activity to the 10 parent molecule. For sTNFR-I, for example, information has been elucidated relevant to sTNFR-I-like molecules (Banner *et al.* (1993), *supra*, and Fu *et al.* (1995), *Protein Engineering*, 8(12):1233-1241). Residues Tyr<sup>9</sup>, Thr<sup>39</sup>, His<sup>55</sup> in Domain 1, residues Phe<sup>49</sup>, Ser<sup>63</sup>, Asp<sup>82</sup> in 15 Domain 2 and residues Tyr<sup>92</sup> and Ser<sup>107</sup> in Domain 3 have been identified as being potentially important for the stabilization of the structure of Domains 1, 2 and 3, respectively. Residues Pro<sup>12</sup> and His<sup>55</sup> have been identified as potentially interacting with Ser<sup>86</sup>-Tyr<sup>87</sup> 20 on subunit C of TNF- $\alpha$ . Residues Glu<sup>45</sup>-Phe<sup>49</sup> have been identified as being in a loop which potentially interacts with residues Leu<sup>29</sup>-Arg<sup>32</sup> of TNF- $\alpha$  subunit A. Residues Gly<sup>48</sup> has been identified as potentially 25 interacting with Asn<sup>19</sup>-Pro<sup>20</sup> on subunit A of TNF- $\alpha$ . Residue His<sup>58</sup>-Leu<sup>60</sup> have been identified as being in an extended strand conformation and side chain interactions with residues Arg<sup>31</sup>-Ala<sup>33</sup> on subunit A of TNF- $\alpha$  have been potentially identified with residue His<sup>58</sup> of sTNFR-I specifically interacting with residue 30 Arg<sup>31</sup>. Residues Lys<sup>64</sup>-Arg<sup>66</sup> have been identified as being in an extended strand conformation and have been identified as having side chain and main chain

interactions with residues Ala<sup>145</sup>-Glu<sup>146</sup> and residue Glu<sup>46</sup> on subunit A of TNF- $\alpha$ . Residue Met<sup>69</sup> has been identified as potentially interacting with residue Tyr<sup>115</sup> on subunit A of TNF- $\alpha$ . Residues His<sup>94</sup>-Phe<sup>101</sup> have been identified as forming a loop which interacts with residues Thr<sup>72</sup>-Leu<sup>75</sup> and Asn<sup>137</sup> of subunit C of TNF- $\alpha$ , with residue Trp<sup>96</sup> of sTNFR-I specifically interacting with residues Ser<sup>71</sup>-Thr<sup>72</sup> on subunit C of TNF- $\alpha$ , Leu<sup>100</sup> of sTNFR-I being in close proximity with residue Asn<sup>137</sup> on subunit C of TNF- $\alpha$  and residue Gln<sup>102</sup> of sTNFR-I specifically interacting with residue Pro<sup>113</sup> on subunit A of TNF- $\alpha$ .

In addition to the cysteines forming the 3 pairs of disulfide bonds within each of the four domains of the molecule, there are several other conserved residues that contribute to the stabilization of the tertiary fold of each domain.

There are two main classes into which these stabilizing residues fall. The first type contributes to the shielding of the disulfide bond sulfur atoms from solvent. An example of this residues in domain 3 is Tyr<sup>92</sup>. In domain 4 Phe<sup>133</sup> helps to shield the Cys<sup>128</sup>-Cys<sup>139</sup> disulfide bond. All four domains have either a Tyr or Phe at these same structurally conserved locations. The second class of stabilizing residues form hydrogen bonds within their respective domains. Within domain 3 Asn<sup>123</sup> and Ser<sup>107</sup> form a hydrogen bond and Ser<sup>107</sup> forms an additional hydrogen bond with Thr<sup>124</sup>. For domain 4 these residues include Asn<sup>144</sup> and Ser<sup>141</sup>.

In addition there are hydrogen bonds between domain 3 and 4 that are not seen between other domains.

These hydrogens bonds are (1) Asn<sup>105</sup> main-chain oxygen and Asn<sup>137</sup> side-chain nitrogen and (2) Ser<sup>107</sup> side-chain oxygen and Asn<sup>137</sup> main-chain nitrogen.

Another useful tool in identifying sites suitable for substitution is molecular modeling. One example of this technique is OPG. Using the homology between OPG and the extracellular ligand binding domains of TNF receptor family members, a three-dimensional model of OPG was generated based upon the known crystal structure of the extracellular domain of TNFR-I (see Example 6). This model was used to identify those residues within OPG which may be important for biological activity. Cysteine residues that are involved in maintaining the structure of the four cysteine-rich domains were identified. The following disulfide bonds were identified in the model: Domain 1: cys41 to cys54, cys44 to cys62, tyr23 and his 66 may act to stabilize the structure of this domain; Domain 2: cys65 to cys80, cys83 to cys98, cys87 to cys105; Domain 3: cys107 to cys118, cys124 to cys142; Domain 4: cys145 to cys160, cys166 to cys185. Residues were also identified which were in close proximity to TNF $\beta$  as shown in Figures 11 and 12A-12B. In this model, it is assumed that OPG binds to a corresponding ligand; TNF $\beta$  was used as a model ligand to simulate the interaction of OPG with its ligand. Based upon this modeling, the following residues in OPG may be important for ligand binding: glu34, lys43, pro66 to gln91 (in particular, pro66, his68, tyr69, tyr70, thr71, asp72, ser73, his76, ser77, asp78, glu79, leu81, tyr82, pro85, val86, lys88, glu90 and gln91), glu153 and ser155.

Alterations in these amino acid residues, either singly or in combination, may alter the biological activity of OPG. For example, changes in specific cysteine residues may alter the structure of individual

cysteine-rich domains, whereas changes in residues important for ligand binding may affect physical interactions of OPG with ligand. Structural models can aid in identifying analogs which have more desirable 5 properties, such as enhanced biological activity, greater stability, or greater ease of formulation.

A skilled artisan will appreciate that initially sites should be modified by substitution in a relatively conservative manner. Such conservative 10 substitutions are shown in Table 1 under the heading of "Preferred Substitutions". If such substitutions result in a change in biological activity, then more substantial changes (Exemplary Substitutions) may be introduced and/or other additions/deletions may be made 15 and the resulting products screened.

TABLE 1: Amino Acid Substitutions

<u>Original Residue</u>	<u>Preferred Substitutions</u>	<u>Exemplary Substitutions</u>
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; norleucine

In making such changes, the hydropathic index of amino acids may be considered. The importance of the 5 hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle (1982), J.

Mol. Biol., 157:105-131, the disclosure of which is incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and 5 still retain a similar biological activity.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the functionally equivalent protein 10 or peptide thereby created is intended for use in immunological embodiments, as in the present case. U.S. Patent 4,554,101, the disclosure of which is incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as 15 governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with a biological property of the protein.

U.S. Patent 4,554,101 also teaches the 20 identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in U.S. Patent 4,554,101 a skilled artisan would be able to identify epitopes, for example, within the amino acid sequence of an 25 STNFR. These regions are also referred to as "epitopic core regions". Numerous scientific publications have been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou and Fasman (1974), 30 Biochemistry, 13(2):222-245; Chou and Fasman (1974), Biochemistry, 13(2):211-222; Chou and Fasman (1978), Adv. Enzymol. Relat. Areas Mol. Biol., 47:45-148; Chou and Fasman (1978), Ann. Rev. Biochem., 47:251-276 and Chou and Fasman (1979), Biophys. J., 26:367-384, the 35 disclosures of which are incorporated herein by reference). Moreover, computer programs are currently

available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson and Wolf (1988), Comput. Appl. Biosci., 5 4(1):181-186 and Wolf et al. (1988), Comput. Appl. Biosci., 4(1):187-191, the disclosures of which are incorporated herein by reference); the program PepPlot® (Brutlag et al. (1990), CABS, 6:237-245 and Weinberger et al. (1985), Science, 228:740-742, the disclosures of 10 which are incorporated herein by reference); and other programs for protein tertiary structure prediction (Fetrow and Bryant (1993), BIOTECHNOLOGY, 11:479-483, the disclosure of which is incorporated herein by reference).

15 In contrast, substantial modifications in the functional and/or chemical characteristics of a parent molecule may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide 20 backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the relative charge or hydrophobicity of the protein at the target site or (c) the bulk of the side chain. Naturally-occurring residues are divided into groups based on 25 common side chain properties:

- 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr;
- 3) acidic: Asp, Glu;
- 4) basic: Asn, Gln, His, Lys, Arg;
- 5) aromatic: Trp, Tyr, Phe; and
- 6) residues that influence chain orientation: Gly, Pro.

30 Non-conservative substitutions may involve the exchange of a member of one of these groups for 35

another. For example, substituted residues may be introduced into regions of OPG or the sTNFRs that are homologous with other NGF/TNF receptor family members or into non-homologous regions of the protein.

5 A variety of amino acid substitutions or deletions may be made to modify or add N-linked or O-linked glycosylation sites, resulting in a protein with altered glycosylation. The sequence may be modified to add glycosylation sites to or to delete N-linked or O-linked glycosylation sites from the parent molecule. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either  
10 Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. In the 30 kDa TNF inhibitor, for example, proven or predicted asparagine residues exist at positions 14, 105 and 111.  
15

Specific mutations of the sequences of the parent molecules may involve substitution of a non-native amino acid at the amino-terminus, carboxy-terminus or at any site of the protein that is modified by the addition of an N-linked or O-linked carbohydrate. Such modifications may be of particular utility in the  
20 addition of an amino acid (e.g., cysteine), which is advantageous for the linking of a water-soluble polymer to form a derivative. For example, WO 92/16221 describes the preparation of sTNFR-I muteins, e.g., wherein an asparagine residue at position 105 of the  
25 native human protein is changed to cysteine (c105 sTNFR-I).  
30

In a specific embodiment, a variant polypeptide will preferably be substantially homologous to the amino acid of the parent molecule from which it is derived. The term "substantially homologous" as used herein means a degree of homology that is in excess of  
35

80%, preferably in excess of 90%, more preferably in excess of 95% or most preferably even 99%. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment, as set forth by Dayhoff (1972), Atlas of Protein Sequence and Structure, 5:124, National Biochemical Research Foundation, Washington, D.C., the disclosure of which is hereby incorporated by reference. Also included within the term "substantially homologous" are variant(s) of parent molecules that may be isolated by cross-reactivity with antibodies to the parent molecule amino acid sequences or whose genes may be isolated through hybridization with the DNA of parent molecules or segments thereof.

#### Polypeptide Derivatives

This invention also comprises chemically modified derivatives of the parent molecule(s) in which the protein is linked to a nonproteinaceous moiety (e.g., a polymer) in order to modify properties. These chemically modified parent molecules are referred to herein as "derivatives". Such derivatives may be prepared by one skilled in the art given the disclosures herein. Conjugates may be prepared using glycosylated, non-glycosylated or de-glycosylated parent molecule(s) and suitable chemical moieties. Typically non-glycosylated parent molecules and water-soluble polymers will be used. Other derivatives encompassed by the invention include post-translational modifications (e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, and chemical modifications of N-linked or O-

linked carbohydrate chains. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

5        Water-soluble polymers are desirable because the protein to which each is attached will not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a  
10      therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically and, if so, the therapeutic profile of the protein (e.g., duration of  
15      sustained release; resistance to proteolysis; effects, if any, on dosage; biological activity; ease of handling; degree or lack of antigenicity and other known effects of a water-soluble polymer on a therapeutic proteins).

20       Suitable, clinically acceptable, water-soluble polymers include but are not limited to polyethylene glycol (PEG), polyethylene glycol propionaldehyde, copolymers of ethylene glycol/propylene glycol, monomethoxy-polyethylene glycol,  
25      carboxymethylcellulose, dextran, polyvinyl alcohol (PVA), polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, poly ( $\beta$ -amino acids) (either homopolymers or random copolymers), poly(n-vinyl  
30      pyrrolidone)polyethylene glycol, polypropylene glycol homopolymers (PPG) and other polyalkylene oxides, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols (POG) (e.g., glycerol) and other polyoxyethylated polyols, polyoxyethylated sorbitol, or polyoxyethylated glucose, colonic acids or  
35

other carbohydrate polymers, Ficoll or dextran and mixtures thereof. As used herein, polyethylene glycol is meant to encompass any of the forms that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

5 Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The water-soluble polymers each may be of any molecular weight and may be branched or unbranched.

10 Generally, the higher the molecular weight or the more branches, the higher the polymer:protein ratio. The water-soluble polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa (the term "about" indicating that in preparations

15 of a water-soluble polymer, some molecules will weigh more, some less, than the stated molecular weight). The average molecular weight of each water-soluble polymer preferably is between about 5 kDa and about 40 kDa, more preferably between about 10kDa and about 35

20 kDa and most preferably between about 15kDa and about 30 kDa.

There are a number of attachment methods available to those skilled in the art, including acylation reactions or alkylation reactions (preferably to generate an amino-terminal chemically modified protein)

25 with a reactive water-soluble molecule. See, for example, EP 0 401 384; Malik *et al.* (1992), Exp. Hematol., 20:1028-1035; Francis (1992), Focus on Growth Factors, 3(2):4-10, published by Mediscript, Mountain

30 Court, Friern Barnet Lane, London N20 0LD, UK; EP 0 154 316; EP 0 401 384; WO 92/16221; WO 95/34326; WO 95/13312; WO 96/11953; WO 96/19459 and WO 96/19459 and the other publications cited herein that relate to pegylation, the disclosures of which are hereby

35 incorporated by reference.

Pegylation also may be specifically carried out using water-soluble polymers having at least one reactive hydroxy group (e.g. polyethylene glycol). The water-soluble polymer can be reacted with an activating group, thereby forming an "activated linker" useful in modifying various proteins. The activated linkers can be monofunctional, bifunctional, or multifunctional.

Activating groups which can be used to link the water-soluble polymer to two or more proteins include the following: sulfone, maleimide, sulfhydryl, thiol, triflate, tresylate, azidirine, oxirane and 5-pyridyl. Useful reagents having a reactive sulfone group that can be used in the methods include, without limitation, chlorosulfone, vinylsulfone and divinylsulfone. These PEG derivatives are stable against hydrolysis for extended periods in aqueous environments at pHs of about 11 or less, and can form linkages with molecules to form conjugates which are also hydrolytically stable. Useful homobifunctional derivatives are PEG-bis-chlorosulfone and PEG-bis-vinylsulfone (see WO 95/13312).

WO 97/04003, the disclosure of which is hereby incorporated by reference, teaches methods of making sulfone-activated linkers by obtaining a compound having a reactive hydroxyl group and converting the hydroxyl group to a reactive Michael acceptor to form an activated linker, with tetrahydrofuran as the solvent for the conversion. The application also teaches a process for purifying the activated linkers which utilizes hydrophobic interaction chromatography to separate the linkers based on size and end-group functionality.

As an example, chemically modified derivatives of OPG may provide such advantages as increased stability, increased time in circulation, or decreased immunogenicity (see U.S. Patent No. 4,179,337). The

chemical moieties for derivitization may be selected from water-soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemically modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

#### Polyvalent Forms

Polyvalent forms, i.e., molecules comprising more than one active moiety, may be constructed. In one embodiment, an sTNFR variant may possess multiple tumor necrosis factor binding sites for the TNF ligand.

Additionally, the molecule may possess at least one tumor necrosis factor binding site and, depending upon the desired characteristic of polyvalent form, at least one site of another molecule (e.g., a TNF- $\alpha$  inhibitor(s), and an OPG).

Active moieties may be linked using conventional coupling techniques (see WO 92/16221, WO 95/13312 and WO 95/34326, the disclosures of which are hereby incorporated by reference). For example, WO 92/16221 and WO 95/34326 describe the preparation of various dimerized sTNFR-I molecules, e.g., dimerized c105 sTNFR-I. Techniques for formation of polyvalent forms include photochemical crosslinking (e.g., exposure to ultraviolet light), chemical crosslinking (e.g., with bifunctional linker molecules such as polyethylene glycol), and mutagenesis (e.g., introduction of additional cysteine residues).

Polyvalent forms may be constructed by chemically coupling at least one parent molecule and another moiety with any clinically accepted linker (e.g., a water-soluble polymer). In principle, the linker must not impart new immunogenicity. The linker also must not, by virtue of the new amino acid residues, alter the hydrophobicity and charge balance of the structure, which affects its biodistribution and clearance. A variety of chemical crosslinkers may be used depending upon which properties of the protein dimer are desired. For example, crosslinkers may be short and relatively rigid or longer and more flexible, may be biologically reversible, and may provide reduced immunogenicity or longer pharmacokinetic half-life.

In one example, OPG molecules are linked through the amino terminus by a two step synthesis (see Example 12). In the first step, OPG is chemically modified at the amino terminus to introduce a protected thiol,

which after purification is deprotected and used as a point of attachment for site-specific conjugation through a variety of crosslinkers with a second OPG molecule. Amino-terminal crosslinks include, but are 5 not limited to, a disulfide bond, thioether linkages using short-chain, bis-functional aliphatic crosslinkers, and thioether linkages to variable length, bifunctional polyethylene glycol crosslinkers (PEG "dumbbells"). Also encompassed by PEG dumbbell 10 synthesis of OPG dimers is a byproduct of such synthesis, termed a "monobell". An OPG monobell consists of a monomer coupled to a linear bifunctional PEG with a free polymer terminus. Alternatively, OPG may be crosslinked directly through a variety of amine 15 specific homobifunctional crosslinking techniques which include reagents such as: diethylenetriaminepentaacetic dianhydride (DTPA), p-benzoquinone (pBQ) or bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>) as well as others known in the art. It is also possible to thiolate OPG 20 directly with reagents such as iminothiolane in the presence of a variety of bifunctional, thiol specific crosslinkers, such as PEG bismaleimide, and achieve dimerization and/or dumbbells in a one step process.

The water-soluble polymers for this polyvalent 25 form can be, based on the monomers listed herein, homopolymers, random or block copolymers, terpolymers straight chain or branched, substituted or unsubstituted. The polymer can be of any length or molecular weight, but these characteristics can affect 30 the biological properties. Polymer average molecular weights particularly useful for decreasing clearance rates in pharmaceutical applications are in the range of 2,000 to 35,000 daltons. In addition, the length of the polymer can be varied to optimize or confer the 35 desired biological activity.

Alternatively, a bivalent molecule may consist of two tandem repeats of parent molecules separated by a polypeptide linker region. The design of the polypeptide linkers is similar in design to the 5 insertion of short loop sequences between domains in the de novo design of proteins (Mutter (1988), TIBS, 13:260-265 and Regan and DeGrado (1988), Science, 241:976-978, the disclosures of which are hereby incorporated by reference). Several different linker 10 constructs have been assembled and shown to be useful for forming single chain antibodies; the most functional linkers vary in size from 12 to 25 amino acids (amino acids having unreactive side groups, e.g., alanine, serine and glycine) which together constitute 15 a hydrophilic sequence, have a few oppositely charged residues to enhance solubility and are flexible (Whitlow and Filpula (1991), Methods: A Companion to Methods in Enzymology, 2:97-105; and Brigido et al. (1993), J. Immunol., 150:469-479, the disclosures of 20 which are hereby incorporated by reference). It has been shown that a linker suitable for single chain antibodies is effective to produce a dimeric form of the human sTNFR-II (Neve et al. (1996), Cytokine, 8(5):365-370, the disclosure of which is hereby 25 incorporated by reference).

Self-associating variants are another example of polyvalent forms. Such self-associating variants may be bound covalently (typically by disulfide bonds) or noncovalently. Analysis of carboxy-terminal deletions 30 of OPG, for example, suggest that at least a portion of the region 186-401 is involved in association of OPG polypeptides. Substitution of part or all of the region of OPG amino acids 186-401 with an amino acid sequence capable of self-association is also encompassed by the 35 invention.

Polyvalent forms may also be formed using substitution variants. Parent molecules may be modified to form dimers or multimers by site-directed mutagenesis to create unpaired cysteine residues for 5 interchain disulfide bond formation.

Additionally, a parent molecule may be chemically coupled to biotin, and the resulting conjugate may then be allowed to bind to avidin, resulting in tetravalent avidin/biotin/parent molecules. A parent molecule may 10 also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugates precipitated with anti-DNP or anti-TNP-IgM to form decameric conjugates.

In yet another embodiment, recombinant fusion 15 proteins may also be produced wherein each recombinant chimeric molecule has a parent molecule(s) sequence amino-terminally or carboxy-terminally fused to all or part of the constant domains, but at least one constant domain, of the heavy or light chain of human 20 immunoglobulin. For example, a chimeric TNF- $\alpha$  inhibitor(s)/IgG1 (or IgG1/TNF- $\alpha$  inhibitor(s)) fusion protein may be produced from a light chain-containing chimeric gene: a TNF- $\alpha$  inhibitor(s)/human kappa light chain chimera (TNF- $\alpha$  inhibitor(s)/Ck) or a human kappa 25 light chain/TNF- $\alpha$  inhibitor(s) chimera (Ck/TNF- $\alpha$  inhibitor(s)); or a heavy chain-containing chimeric gene: a TNF- $\alpha$  inhibitor(s)/human gamma-1 heavy chain chimera (TNF- $\alpha$  inhibitor(s)/Cg-1) or a human gamma-1 heavy chain/TNF- $\alpha$  inhibitor(s) chimera (Cg-1/TNF- $\alpha$  30 inhibitor(s)). Alternatively, an OPG-Fc chimera may be formed as described in WO 97/23614, which is hereby incorporated by reference. Following transcription and translation of a heavy-chain chimeric gene, or of a light chain-containing gene and a heavy-chain chimeric

gene, the gene products may be assembled into a single chimeric molecule having a parent molecule(s) displayed bivalently. Additional details relating to the construction of such chimeric molecules are disclosed  
5 in United States Patent 5,116,964, WO 89/09622, WO 91/16437, WO 97/23614 and EP 315062, the disclosures of which are hereby incorporated by reference.

In yet a further embodiment, recombinant fusion proteins may also be produced wherein each recombinant  
10 chimeric molecule has at least one TNF- $\alpha$  inhibitor(s), as described herein, and at least a portion of the region 186-401 of osteoprotogerin or a variant thereof, as described in European Patent Application No. 96309363.8, the disclosures of which are hereby  
15 incorporated by reference. Either the TNF- $\alpha$  inhibitor(s) or the portion of osteoprotogerin may be at the amino-terminus or the carboxy-terminus of the chimeric molecule.

#### Nucleic Acids

20 The invention provides for an isolated nucleic acid encoding a polypeptide having at least one of the biological activities of OPG. As described herein, the biological activities of OPG include, but are not limited to, any activity involving bone metabolism and  
25 in particular, include increasing bone density. The nucleic acids of the invention are selected from the following:

- a) the nucleic acid sequences as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) or complementary strands thereof;
- 30 b) the nucleic acids which hybridize under stringent conditions with the polypeptide-encoding region in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124); and

c) nucleic acids which hybridize under stringent conditions with nucleotides 148 through 337 inclusive as shown in Figure 1A.

5 d) the nucleic acid sequences which are degenerate to the sequences in (a) and (b).

The invention provides for nucleic acids which encode rat, mouse and human OPG as well as nucleic acid sequences hybridizing thereto which encode a polypeptide having at least one of the biological 10 activities of OPG. Also provided for are nucleic acids which hybridize to a rat OPG EST encompassing nucleotides 148-337 as shown in Figure 1A. The conditions for hybridization are generally of high stringency such as 5xSSC, 50% formamide and 42°C 15 described in Example 1 of the specification. Equivalent stringency to these conditions may be readily obtained by adjusting salt and organic solvent concentrations and temperature. The nucleic acids in (b) encompass sequences encoding OPG-related polypeptides which do 20 not undergo detectable hybridization with other known members of the TNF receptor superfamily. In a preferred embodiment, the nucleic acids are as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124)..

25 The length of hybridizing nucleic acids of the invention may be variable since hybridization may occur in part or all of the polypeptide-encoding regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), and may also occur 30 in adjacent noncoding regions. Therefore, hybridizing nucleic acids may be truncations or extensions of the sequences shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124). Truncated or extended nucleic acids are encompassed by the 35 invention provided they retain one or more of the biological properties of OPG. The hybridizing nucleic

acids may also include adjacent noncoding regions which are 5' and/or 3' to the OPG coding region. The noncoding regions include regulatory regions involved in OPG expression, such as promoters, enhancers,  
5 translational initiation sites, transcription termination sites and the like.

Hybridization conditions for nucleic acids are described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed. Cold Spring Harbor Laboratory  
10 Press, Cold Spring Harbor, New York (1989)

DNA encoding rat OPG was provided in plasmid pMO-B1.1 deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under ATCC accession no. 69970. DNA encoding mouse OPG was  
15 provided in plasmid pRcCMV-murine OPG deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under accession no. 69971. DNA encoding human OPG was provided in plasmid pRcCMV - human OPG deposited with the American Type Culture  
20 Collection, Rockville, MD on December 27, 1995 under accession no. 69969. The nucleic acids of the invention will hybridize under stringent conditions to the DNA inserts of ATCC accession nos. 69969, 69970, and 69971 and have at least one of the biological activities of  
25 OPG.

Also provided by the invention are derivatives of the nucleic acid sequences as shown in Figures 2B, 9A and 9B. As used herein, derivatives include nucleic acid sequences having addition, substitution, insertion or deletion of one or more residues such that the resulting sequences encode polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting polypeptide has the activity of OPG. The nucleic acid  
30 derivatives may be naturally occurring, such as by splice variation or polymorphism, or may be constructed  
35

using site-directed mutagenesis techniques available to the skilled worker. One example of a naturally occurring variant of OPG is a nucleic acid encoding a lys to asn change at residue 3 within the leader sequence (see Example 5). It is anticipated that nucleic acid derivatives will encode amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other derivatives include a nucleic acid encoding a membrane-bound form of OPG having an extracellular domain as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) along with transmembrane and cytoplasmic domains.

In one embodiment, derivatives of OPG include nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the carboxy terminus. Nucleic acids encoding OPG may have from 1 to 216 amino acids deleted from the carboxy terminus. Optionally, an antibody Fc region may extend from the new carboxy terminus to yield a biologically active OPG-Fc fusion polypeptide. (see Example 11). In preferred embodiments, nucleic acids encode OPG having the amino acid sequence from residues 22-185, 22-189, 22-194 or 22-201 (using numbering in Figure 9E-F) and optionally, encoding an Fc region of human IgG.

Also included are nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the amino terminus. Truncated forms include those lacking part or all the 21 amino acids comprising the leader sequence. Additionally, the invention provides for nucleic acids encoding OPG having from 1 to 10 amino acids deleted from the mature amino terminus (at residue 22) and ,optionally, having from 1 to 216 amino acids deleted from the carboxy terminus (at residue 401). Optionally, the nucleic acids may encode a methionine residue at the amino terminus. Examples of

such OPG truncated polypeptides are described in Example 8.

Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing OPG. In humans, tissue sources for OPG include kidney, liver, placenta and heart. Genomic DNA encoding OPG is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to reconstitute part or all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of interferon genes). RNA is obtained most easily by prokaryotic expression vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Nucleic acid sequences of the invention are used for the detection of OPG sequences in biological samples in order to determine which cells and tissues are expressing OPG mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to OPG. Such screening is well within the capabilities of one skilled in the art using appropriate hybridization conditions to detect homologous sequences. The nucleic acids are also useful for modulating the expression of OPG levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity (see Example 3).

#### Vectors and Host Cells

Expression vectors containing nucleic acid sequences encoding OPG, host cells transformed with

said vectors and methods for the production of OPG are also provided by the invention. An overview of expression of recombinant proteins is found in Methods of Enzymology v. 185, Goeddel, D.V. ed. Academic Press 5 (1990).

- Host cells for the production of OPG include prokaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression.
- 10 Preferred mammalian host cells include COS, CHOD-, 293, CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for OPG activity.
- 15 Mammalian expression allows for the production of secreted polypeptides which may be recovered from the growth medium.

Vectors for the expression of OPG contain at a minimum sequences required for vector propagation and 20 for expression of the cloned insert. These sequences include a replication origin, selection marker, promoter, ribosome binding site, enhancer sequences, RNA splice sites and transcription termination site. Vectors suitable for expression in the aforementioned 25 host cells are readily available and the nucleic acids of the invention are inserted into the vectors using standard recombinant DNA techniques. Vectors for tissue-specific expression of OPG are also included. Such vectors include promoters which function 30 specifically in liver, kidney or other organs for production in mice, and viral vectors for the expression of OPG in targeted human cells.

Using an appropriate host-vector system, OPG is produced recombinantly by culturing a host cell 35 transformed with an expression vector containing nucleic acid sequences encoding OPG under conditions

such that OPG is produced, and isolating the product of expression. OPG is produced in the supernatant of transfected mammalian cells or in inclusion bodies of transformed bacterial host cells. OPG so produced may 5 be purified by procedures known to one skilled in the art as described below. The expression of OPG in mammalian and bacterial host systems is described in Examples 7 and 8. Expression vectors for mammalian hosts are exemplified by plasmids such as pDSR $\alpha$  10 described in PCT Application No. 90/14363. Expression vectors for bacterial host cells are exemplified by plasmids pAMG21 and pAMG22-His described in Example 8. Plasmid pAMG21 was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 15 under accession no. 98113. Plasmid pAMG22-His was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98112. It is anticipated that the specific plasmids and host cells described are for illustrative purposes and 20 that other available plasmids and host cells could also be used to express the polypeptides.

The invention also provides for expression of OPG from endogenous nucleic acids by in vivo or ex vivo recombination events to allow modulation of OPG from 25 the host chromosome. Expression of OPG by the introduction of exogenous regulatory sequences (e.g. promoters or enhancers) capable of directing the production of OPG from endogenous OPG coding regions is also encompassed. Stimulation of endogenous regulatory 30 sequences capable of directing OPG production (e.g. by exposure to transcriptional enhancing factors) is also provided by the invention.

Antibodies

Also encompassed by the invention are antibodies 35 specifically binding to OPG. Antigens for the generation of antibodies may be full-length

polypeptides or peptides spanning a portion of the OPG sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with OPG are known to one skilled in the art (see, for example, Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the art. Also encompassed are human antibodies made in mice.

Anti-OPG antibodies of the invention may be used as an affinity reagent to purify OPG from biological samples (see Example 10). In one method, the antibody is immobilized on CnBr-activated Sepharose and a column of antibody-Sepharose conjugate is used to remove OPG from liquid samples. Antibodies are also used as diagnostic reagents to detect and quantitate OPG in biological samples by methods described below.

Pharmaceutical compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of a polypeptide comprising OPG or the other therapeutic molecules used (e.g., IL-1ra, STNF-RI, or SLPI) together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. Two or more of the therapeutic

molecules (e.g., OPG, IL-1ra, sTNF-RI, or SLPI) can be formulated together or packaged together in a kit. The term "therapeutically effective amount" means an amount which provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal or benzyl alcohol, and antioxidants such as ascorbic acid or sodium metabisulfite. Also encompassed are compositions comprising any of the therapeutic molecules modified with water-soluble polymers to increase solubility or stability. Compositions may also comprise incorporation of any of the therapeutic molecules into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time.

Specifically, compositions herein may comprise incorporation into polymer matrices such as hydrogels, silicones, polyethylenes, ethylene-vinyl acetate copolymers, or biodegradable polymers. Examples of hydrogels include polyhydroxyalkylmethacrylates (p-HEMA), polyacrylamide, polymethacrylamide, polyvinylpyrrolidone, polyvinyl alcohol and various polyelectrolyte complexes. Examples of biodegradable polymers include polylactic acid (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA, polyamides and copolymers of polyamides and polyesters. Other controlled release formulations include microcapsules, microspheres, macromolecular complexes and polymeric beads which may be administered by injection.

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the

pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18<sup>th</sup> ed. A.R. Gennaro, ed. Mack, Easton, PA 5 (1980).

Compositions of the invention may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the therapeutic molecule coding region to cells and tissues as part of an anti-sense or gene therapy regimen.

20 Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, 25 osteocytes, osteoblasts and osteoclasts, are involved in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of bone tissue whereas osteoclasts are associated with resorption. Resorption, or the dissolution of bone 30 matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal tissue and in resorption induced by hormones. For 35 instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing

concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to 5 parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone formation and bone resorption. Peak bone mass occurs after skeletal maturity prior to the fourth decade. 10 Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts earlier in females than males and is distinctly accelerated after menopause in some females 15 (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels. Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone 20 destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in 25 bone resorption with a normal rate of bone formation. About 25 to 30 percent of all white females in the United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric 30 fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been 35 identified which may contribute to the condition. They include alteration in hormone levels accompanying aging

and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard 5 the process. To date, however, an effective treatment for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of OPG. The bone disorder may be any disorder 10 characterized by a net bone loss (osteopenia or osteolysis). In general, treatment with OPG is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption 15 rate is above normal or to reduce bone resorption to below normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with OPG include the following:

- 20 • Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.
- 25 • Paget's disease of bone (osteitis deformans) in adults and juveniles
- 30 • Osteomyelitis, or an infectious lesion in bone, leading to bone loss.
- Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignancies (multiple myeloma, lymphoma and leukemia), idiopathic hypercalcemia, and hypercalcemia

associated with hyperthyroidism and renal function disorders.

- Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic and renal diseases.
- Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus, rheumatoid arthritis, periodontal disease, osteolytic metastasis, and other conditions

It is understood that OPG may be used alone or in conjunction with other factors for the treatment of bone disorders. In one embodiment, osteoprotegerin is used in conjunction with a therapeutically effective amount of a factor which stimulates bone formation. Such factors include but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12; transforming growth factor- $\beta$  (TGF- $\beta$ ) and TGF- $\beta$  family members; interleukin-1 (IL-1) inhibitors; TNF $\alpha$  inhibitors; parathyroid hormone and analogs thereof, parathyroid related protein and analogs thereof; E series prostaglandins; bisphosphonates (such as alendronate and others); bone-enhancing minerals such as fluoride and calcium; non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors, such as Celebrex™ and Vioxx™; immunosuppressants, such as methotrexate or leflunomide; serine protease inhibitors such as secretory leukocyte protease inhibitor (SLPI); IL-6 inhibitors (e.g., antibodies to IL-6), IL-8 inhibitors (e.g., antibodies to IL-8); IL-18 inhibitors (e.g., IL-18 binding protein or IL-18 antibodies); Interleukin-1 converting enzyme (ICE) modulators; fibroblast growth

factors FGF-1 to FGF-10 and FGF modulators; PAF antagonists; keratinocyte growth factor (KGF), KGF-related molecules, or KGF modulators; matrix metalloproteinase (MMP) modulators; Nitric oxide synthase (NOS) modulators, including modulators of inducible NOS; modulators of glucocorticoid receptor; modulators of glutamate receptor; modulators of lipopolysaccharide (LPS) levels; and noradrenaline and modulators and mimetics thereof.

10       The invention also relates to treatment of IL-1 mediated disease by treatment with an IL-1 inhibitor in conjunction with a serine protease inhibitor. In particular, this method is useful for treatment of asthma and rheumatoid arthritis.

15       The invention relates further to treatment of TNF-mediated disease by treatment with a TNF inhibitor in conjunction with a serine protease inhibitor. In particular, this method is useful for treatment of rheumatoid arthritis.

20       In preferred embodiments, a polypeptide comprising OPG is used in conjunction with particular therapeutic molecules to treat various inflammatory conditions, autoimmune conditions, and other conditions leading to bone loss. Depending on the condition and the desired level of treatment, two, three, or more agents may be administered. These agents may be provided together by inclusion in the same formulation or inclusion in a treatment kit, or they may be provided separately. When administered by gene therapy, the genes encoding the protein agents may be included in the same vector, optionally under the control of the same promoter region, or in separate vectors. Particularly preferred molecules in the aforementioned classes are as follows.

- IL-1 inhibitors: IL-1ra proteins and soluble IL-1 receptors. The most preferred IL-1 inhibitor is anakinra.
- TNF- $\alpha$  inhibitors: soluble tumor necrosis factor receptor type I (sTNF-RI; -RI is also called the p55 receptor); soluble tumor necrosis factor receptor type II (also called the p75 receptor); and monoclonal antibodies that bind the TNF receptor. Most preferred is sTNF-RI as described in WO 98/24463, etanercept (Enbrel $\circledast$ ), and Avakine $\circledast$ . Exemplary TNF- $\alpha$  inhibitors are described in EP 422 339, EP 308 378, EP 393 438, EP 398 327, and EP 418 014.
- serine protease inhibitors: SLPI, ALP, MPI, HUSI-I, BMI, and CUSI. These inhibitors also may be viewed as exemplary LPS modulators, as SLPI has been shown to inhibit LPS responses. Jin *et al.* (1997), *Cell* 88(3): 417-26 (incorporated by reference).

Particularly preferred methods of treatment concern use of TNF- $\alpha$  inhibitors and IL-1 inhibitors in conjunction with polypeptides comprising OPG. Such polypeptides may be used with either or both TNF- $\alpha$  inhibitors and IL-1 inhibitors for treatment of conditions such as rheumatoid arthritis and multiple sclerosis.

The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

30

#### EXAMPLE 1

##### Identification and isolation of the rat OPG cDNA

Materials and methods for cDNA cloning and analysis are described in Maniatis *et al.*, *ibid*. Polymerase chain reactions (PCR) were performed using a Perkin-Elmer 9600 thermocycler using PCR reaction mixture (Boehringer-Mannheim) and primer concentrations

specified by the manufacturer. In general, 25-50  $\mu$ l reactions were denatured at 94°C, followed by 20-40 cycles of 94°C for 5 seconds, 50-60°C for 5 seconds, and 72°C for 3-5 minutes. Reactions were then treated 5 for 72 °C for 3-5 minutes. Reactions were then analyzed by gel electrophoresis as described in Maniatis *et al.*, ibid.

A cDNA library was constructed using mRNA isolated from embryonic d20 intestine for EST analysis (Adams *et* 10 *al.* *Science* **252**, 1651-1656 (1991)). Rat embryos were dissected, and the entire developing small and large intestine removed and washed in PBS. Total cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi *Anal.* 15 *Biochem.* **162**, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the manufacturer's recommended procedures. A random primed cDNA library was prepared 20 using the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md). The random cDNA primer containing an internal Not I restriction site was used to initiate first strand synthesis and had the following sequence:

5'-AAAGGAAGGAAAAACGGCCGCTACANNNNNNNNT-3'

25 (SEQ ID NO:1)

Not I

For the first strand synthesis three separate reactions were assembled that contained 2.5  $\mu$ g of poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random 30 primer. After second strand synthesis, the reaction products were separately extracted with a mixture of phenol:choroform:isoamyl alcohol (25:24:1 ratio), and then ethanol precipitated. The double strand (ds) cDNA products of the three reactions were combined and 35 ligated to the following ds oligonucleotide adapter:

5'-TCGACCCACCGGTCCG-3' (SEQ ID NO:2)

3'-GGGTGCAGGCp-5' (SEQ ID NO:3)

After ligation the cDNA was digested to completion with Not I, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated. The resuspended cDNA was then size fractionated by gel filtration using premade columns provided with the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) as recommended by the manufacturer. The two fractions containing the largest cDNA products were pooled, ethanol precipitated and then directionally ligated into Not I and Sal I digested pMOB vector DNA (Strathmann *et al*, 1991). The ligated cDNA was introduced into competent ElectroMAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) by electroporation. For automated sequence analysis approximately 10,000 transformants were plated on 20cm x 20cm agar plates containing ampicillin supplemented LB nutrient media. The colonies that arose were picked and arrayed onto 96 well microtiter plates containing 200 ml of L-broth, 7.5% glycerol, and 50 µg/ml ampicillin. The cultures were grown overnight at 37°C, a duplicate set of microtiter plates were made using a sterile 96 pin replicating tool, then both sets were stored at -80°C for further analysis. For full-length cDNA cloning approximately one million transformants were plated on 96 bacterial ampicillin plates containing about 10,000 clones each. The plasmid DNA from each pool was separately isolated using the Qiagen Plasmid Maxi Kit (Qiagen Corp., Germany) and arrayed into 96 microtiter plates for PCR analyses.

To sequence random fetal rat intestine cDNA clones, glycerol stocks were thawed, and small aliquots diluted 1:25 in distilled. Approximately 3.0 ul of diluted bacterial cultures were added to PCR reaction

mixture (Boehringer-Mannheim) containing the following oligonucleotides:

5' -TGTAAAACGACGGCCAGT-3' (SEQ ID NO:4)

5' -CAGGAAACAGCTATGACC-3' (SEQ ID NO:5)

5       The reactions were incubated in a thermocycler (Perkin-Elmer 9600) with the following cycle conditions: 94°C for 2 minutes; 30 cycles of 94°C for 5 seconds, 50°C for 5 seconds, and 72°C for 3 minutes.; 72°C for 4 minutes. After incubation in the 10 thermocycler, the reactions were diluted with 2.0 mL of water. The amplified DNA fragments were further purified using Centricon columns (Princeton Separations) using the manufacturer's recommended procedures. The PCR reaction products were sequenced on 15 an Applied Biosystems 373A automated DNA sequencer using T3 primer (oligonucleotide 353-23; 5' - CAATTAACCCTCACTAAAGG-3') (SEQ ID NO:6) Taq dye-terminator reactions (Applied Biosystems) following the manufacturer's recommended procedures.

20       The resulting 5' nucleotide sequence obtained from randomly picked cDNA clones translated and then compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson *et al.* Meth. Enzymol. 183, (1990)).

25       Translated sequences were also analysed for the presence of a specific cysteine-rich protein motif found in all known members of the tumor necrosis factor receptor (TNFR) superfamily (Smith *et al.* (1994) Cell 76: 959-62), using the sequence profile method of Gribskov *et al.* (1987), Proc. Natl. Acad. Sci. USA 83: 4355-9), as modified by Luethy *et al.* (1994), Protein Science 3: 139-46.

30       Using the FASTA and Profile search data, an EST, FRI-1 (Fetal Rat Intestine-1), was identified as a 35 possible new member of the TNFR superfamily. FRI-1

contained an approximately 600 bp insert with a LORF of about 150 amino acids. The closest match in the database was the human type II TNFR (TNFR-II). The region compared showed an about 43% homology between 5 TNFR-II and FRI-1 over this 150 aa LORF. Profile analysis using the first and second cysteine-rich repeats of the TNFR superfamily yielded a Z score of about 8, indicating that the FRI-1 gene possibly encodes a new family member.

10 To deduce the structure of the FRI-1 product, the fetal rat intestine cDNA library was screened for full length clones. The following oligonucleotides were derived from the original FRI-1 sequence:

15 5'-GCATTATGACCCAGAAACCGGAC-3' (SEQ ID NO:7)  
5'-AGGTAGGCCCTTCCTCACATTC-3' (SEQ ID NO:8)

These primers were used in PCR reactions to screen 96 pools of plasmid DNA, each pool containing plasmid DNA from 10,000 independent cDNA clones. Approximately 1 ug of plasmid pool DNA was amplified in a PCR 20 reaction mixture (Boehringer-Mannheim) using a Perkin-Elmer 96 well thermal cycler with the following cycle conditions: 2 min at 94°C, 1 cycle; 15 sec at 94°C, then 45 sec at 65°C, 30 cycles; 7 min at 65°C, 1 cycle. PCR reaction products were analysed by gel electrophoresis. 25 13 out of 96 plasmid DNA pools gave rise to amplified DNA products with the expected relative molecular mass.

DNA from one positive pool was used to transform 30 competent ElectroMAX DH10B *E. coli* (Gibco BRL, Gaithersburg, MD) as described above. Approximately 40,000 transformants were plated onto sterile nitrocellulose filters (BA-85, Schleicher and Schuell), and then screened by colony hybridization using a <sup>32</sup>P-dCTP labeled version of the PCR product obtained above. Filters were prehybridized in 5X SSC, 50% deionized 35 formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C.

Filters were then hybridized in 5X SSC, 50% deionized formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and about 5 ng/ml of labelled probe for about 18 hours at 42°C. The filters  
5 were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 minutes at 55°C, and finally in 0.5X SSC for 10-15 min at 55°C. Hybridizing clones were detected following autoradiography, and then replated onto nitrocellulose filters for secondary screening. Upon secondary  
10 screening, a plasmid clone (pB1.1) was isolated, then amplified in L-broth media containing 100 ug/ml ampicillin and the plasmid DNA obtained. Both strands of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA  
15 search of the public database to detect any existing sequence matches and/or similarities. No matches to any known genes or EST's were found, although there was an approximate 45% similarity to the human and mouse TNFR-II genes. A methionine start codon is found at bp 124  
20 of the nucleotide sequence, followed by a LORF encoding 401 aa residues that terminates at bp 1327. The 401 aa residue product is predicted to have a hydrophobic signal peptide of approximately 31 residues at its N-terminus, and 4 potential sites of N-linked  
25 glycosylation. No hydrophobic transmembrane spanning sequence was identified using the PepPlot program (Wisconsin GCG package, version 8.1). The deduced 401 aa sequence was then used to search the protein database. Again, there were no existing matches,  
30 although there appeared to be a strong similarity to many members of the TNFR superfamily, most notably the human and mouse TNFR-II. A sequence alignment of this novel protein with known members of the TNFR-superfamily was prepared using the Pileup program, and  
35 then modified by PrettyPlot (Wisconsin GCG package,

version 8.1). This alignment shows a clear homology between the full length FRI-1 gene product and all other TNFR family members. The homologous region maps to the extracellular domain of TNFR family members, and 5 corresponds to the three or four cysteine-rich repeats found in the ligand binding domain of these proteins. This suggested that the FRI-1 gene encoded a novel TNFR family member. Since no transmembrane spanning region was detected we predicted that this may be a secreted 10 receptor, similar to TNFR-I derived soluble receptors (Kohno *et al.* (1990), *Proc. Natl. Acad. Sci. USA* 87: 8331-5). Due to the apparent biological activity of the FRI-1 gene (*vide infra*), the product was named Osteoprotegerin (OPG).

15

#### EXAMPLE 2

##### OPG mRNA Expression Patterns in Tissues

Multiple human tissue northern blots (Clonetech) were probed with a  $^{32}\text{P}$ -dCTP labelled FRI-1 PCR product to detect the size of the human transcript and to 20 determine patterns of expression. Northern blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS, and 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA for 2-4 hr at 42°C. The blots were then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's 25 solution, 0.1% SDS, 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C. The blots were then washed in 2X SSC for 10 min at room temperature, 1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15 min.

30

Using a probe derived from the rat gene, a predominant mRNA species with a relative molecular mass of about 2.4 kb is detected in several tissues, including kidney, liver, placenta, and heart. Highest levels are detected in the kidney. A large mRNA species 35 of Mr 4.5 and 7.5 kb was detected in skeletal muscle

and pancreas. In human fetal tissue, kidney was found to express relatively high levels of the 2.4 kb mRNA. Using a human probe (vide infra), only the 2.4 kb transcript is detected in these same tissues. In 5 addition, relatively high levels of the 2.4 kb transcript was detected in the lymph node, thymus, spleen and appendix. The size of the transcript detected by both the rat and human Osteoprotegerin gene is almost identical to the length of the rat pB1.1 10 FRI-1 insert, suggesting it was a full length cDNA clone.

**EXAMPLE 3**

Systemic delivery of OPG in transgenic mice

The rat OPG clone pb1.1 was used as template to 15 PCR amplify the coding region for subcloning into an ApoE-liver specific expression vector (Simonet *et al.* J. Clin. Invest. 94, 1310-1319 (1994), and PCT Application No. US94/11675 and co-owned U.S. Serial No. 08/221,767. The following 5' and 3' oligonucleotide 20 primers were used for PCR amplification, respectively:

5'-GACTAGTCCCCACAATGAACAAAGTGGCTGTG-3'  
(SEQ ID NO:9)  
5'-ATAAGAATGCAGCCGCTAAACTATGAAACAGCCCAGTGACCATTTC-3'  
(SEQ ID NO:10)

25 The PCR reaction mixture (Boehringer-Mannheim) was treated as follows: 94°C for 1 minute, 1 cycle; 94°C for 20 sec, 62°C for 30 sec, and 74 C for 1 minute, 25 cycles. Following amplification, the samples were purified over Qiagen PCR columns and digested overnight 30 with SpeI and NotI restriction enzymes. The digested products were extracted and precipitated and subcloned into the ApoE promoter expression vector. Prior to microinjecting the resulting clone, HE-OPG, it was sequenced to ensure it was mutation-free.

35 The HE-OPG plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified

plasmid DNA was digested with XhoI and Ase I, and the 3.6 kb transgene insert was purified by gel electrophoresis. The purified fragment was diluted to a stock injection solution of 1 µg/ml in 5 mM Tris, pH 5 7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x BDF1-bred mice were injected essentially as described (Brinster *et al.* (1985), Proc. Natl. Acad. Sci. USA 82: 4338), except that injection needles were beveled and siliconized before use. Embryos were cultured overnight 10 in a CO<sub>2</sub> incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

Following term pregnancy, 49 offspring were obtained from implantation of microinjected embryos. 15 The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the human Apo E intron which was included in the expression vector. The oligos used for PCR amplification were:

20 5'- GCC TCT AGA AAG AGC TGG GAC-3' (SEQ ID NO:11)  
5'- CGC CGT GTT CCA TTT ATG AGC-3' (SEQ ID NO:12)

The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were 25 identified as PCR positive transgenic founders.

At 8-10 weeks of age, five transgenic founders (2, 11, 16, 17, and 28) and five controls (1, 12, 15, 18, and 30) were sacrificed for necropsy and pathological analysis. Liver was isolated from the remaining 4 30 founders by partial hepatectomy. For partial hepatectomy, the mice were anesthetized and a lobe of liver was surgically removed. Total cellular RNA was isolated from livers of all transgenic founders, and 5 negative control littermates as described (McDonald *et* 35 *al.* Meth. Enzymol. 152, 219 (1987)). Northern blot

analysis was performed on these samples to assess the level of transgene expression. Approximately 10ug of total RNA from each animal liver was resolved by electrophoresis denaturing gels (Ogden *et al.* Meth. Enzymol 152, 61 (1987)), then transferred to HYBOND-N nylon membrane (Amersham), and probed with <sup>32</sup>P dCTP-labelled pB1.1 insert DNA. Hybridization was performed overnight at 42°C in 50% Formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution, 100 µg/ml denatured salmon sperm DNA and 2-4 x 10<sup>6</sup> cpm of labeled probe/ml of hybridization buffer. Following hybridization, blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each, and then twice in 0.1 x SSC, 0.1% SDS at 55°C for 5-10 min each. Expression of the transgene in founder and control littermates was determined following autoradiography.

The northern blot data indicate that 7 of the transgenic founders express detectable levels of the transgene mRNA (animal #'s 2,11,16,17,22,33, and 45). The negative control mice and one of the founders (#28) expressed no transgene-related mRNA. Since OPG is predicted to be a secreted protein, overexpression of transgene mRNA should be a proxy for the level of systemically delivered gene product. Of the PCR and northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

**EXAMPLE 4**

30           Biological activity of OPG

Five of the transgenic mice (animals 2,11,16,17 and 28) and 5 control littermates (animals 1,12,15,18, and 30) were sacrificed for necropsy and pathological analysis using the following procedures:

Prior to euthanasia, all animals had their identification numbers verified, then were weighed, anesthetized and blood drawn. The blood was saved as both serum and whole blood for a complete serum chemistry and hematology panel. Radiography was performed just after terminal anesthesia by lethal CO<sub>2</sub> inhalation, and prior to the gross dissection.

Following this, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected included the liver, spleen, pancreas, stomach, duodenum, ileum, colon, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, eosphagus, thyroid, jejunem, cecum, rectum, adrenals, urinary bladder, and skeletal muscle. Prior to fixation the whole organ weights were determined for the liver, stomach, kidney, adrenals, spleen, and thymus. After fixation the tissues were processed into paraffin blocks, and 3 um sections were obtained. Bone tissue was decalcified using a formic acid solution, and all sections were stained with hematoxylin and eosin. In addition, staining with Gomori's reticulin and Masson's trichrome were performed on certain tissues. Enzyme histochemistry was performed to determine the expression of tartrate resistant acid phosphatase (TRAP), an enyzme highly expressed by osteoclasts, multinucleated bone-resorbing cells of monocyte-macrophage lineage.

Immunohistochemistry for BrdU and F480 monocyte-macrophage surface antigen was also performed to detect replicating cells and cells of the monocyte-macrophage lineage, respectively. To detect F480 surface antigen expression, formalin fixed, paraffin embedded 4 $\mu$ m sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-

mouse F480 (Harlan, Indianapolis, IN). This antibody was detected by biotinylated rabbit anti-rat immunoglobulins, peroxidase conjugated strepavidin (BioGenex San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with hematoxylin.

Upon gross dissection and observation of visceral tissues, no abnormalities were found in the transgene expressors or control littermates. Analysis of organ weight indicate that spleen size increased by approximately 38% in the transgenic mice relative to controls. There was a slight enlargement of platelet size and increased circulating unstained cells in the transgene expressors. There was a marginal decrease in platelet levels in the transgene expressors. In addition, the serum uric acid, urea nitrogen, and alkaline phosphatase levels all trended lower in the transgene expressors. The expressors were found to have increased radiodensity of the skeleton, including long bones (femurs), vertebrae, and flat bones (pelvis). The relative size of femurs in the expressors were not different from the control mice.

Histological analysis of stained sections of bone from the OPG expressors show severe osteopetrosis with the presence of cartilage remnants from the primary spongiosa seen within bone trabeculae in the diaphysis of the femur. A clearly defined cortex was not identifiable in the sections of femur. In normal animals, the central diaphysis is filled with bone marrow. Sections of vertebra also show osteopetrotic changes implying that the OPG-induced skeletal changes were systemic. The residual bone marrow showed predominantly myeloid elements. Megakaryocytes were present. Reticulin stains showed no evidence for reticulin deposition. Immunohistochemistry for F480, a cell surface antigen expressed by cells of monocyte-

macrophage derivation in the mouse, showed the presence of F480 positive cells in the marrow spaces. Focally, flattened F480 positive cells could be seen directly adjacent to trabecular bone surfaces.

5       The mesenchymal cells lining the bony trabeculae were flattened and appeared inactive. Based on H&E and TRAP stains, osteoclasts were rarely found on the trabecular bone surfaces in the OPG expressors. In contrast, osteoclasts and/or chondroclasts were seen in 10 the region of the growth plate resorbing cartilage, but their numbers may be reduced compared to controls. Also, osteoclasts were present on the cortical surface of the metaphysis where modelling activity is usually robust. The predominant difference between the 15 expressors and controls was the profound decrease in trabecular osteoclasts, both in the vertebrae and femurs. The extent of bone accumulation was directly correlated with the level of OPG transgene mRNA detected by northern blotting of total liver RNA.

20       The spleens from the OPG expressors had an increased amount of red pulp with the expansion due to increased hematopoiesis. All hematopoietic lineages are represented. F480 positive cells were present in both control and OPG expressors in the red pulp. Two of the 25 expressors (2 and 17) had foci of extramedullary hematopoiesis within the liver and this is likely due to the osteopetrotic marrow.

There were no observable abnormalities in the thymus, lymph nodes, gastrointestinal tract, pancreatico- 30 hepatobiliary tract, respiratory tract, reproductive system, genito-urinary system, skin, nervous system, heart and aorta, breast, skeletal muscle and fat.

**EXAMPLE 5**

**Isolation of mouse and human OPG cDNA**

35       A cDNA clone corresponding to the 5' end of the mouse OPG mRNA was isolated from a mouse kidney cDNA

library (Clontech) by PCR amplification. The oligonucleotides were derived from the rat OPG cDNA sequence and are shown below:

- 5 5'-ATCAAAGGCAGGGCATACTCCTG-3' (SEQ ID NO:13)  
5'-GTTGCACTCCTGTTCACGGTCTG-3' (SEQ ID NO:14)  
5'-CAAGACACCTTGAAGGGCCTGATG-3' (SEQ ID NO:15)  
5'-TAACTTTACAGAAAGAGCATCAGC-3' (SEQ ID NO:16)
- 10 5'-AGCGCGGCCGCATGAACAAGTGGCTGTGCTGCG-3' (SEQ ID NO:17)  
5'-AGCTCTAGAGAACAGCCCAGTGACCATTCC-3' (SEQ ID NO:18)
- The partial and full-length cDNA products obtained in this process were sequenced. The full-length product was digested with Not I and XbaI, then directionally cloned into the plasmid vector pRcCMV (Invitrogen). The 15 resulting plasmid was named pRcCMV-Mu-OPG. The nucleotide sequence of the cloned product was compared to the rat OPG cDNA sequence. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 88% identical. The mouse cDNA 20 sequence contained a 401 aa LORF, which was compared to the rat OPG sequence and found to be about 94% identical without gaps. This indicates that the mouse cDNA sequence isolated encodes the murine OPG, and that the sequence and structure has been highly conserved 25 throughout evolution. The mouse OPG sequence contains an identical putative signal peptide at its N-terminus, and all 4 potential sites of N-linked glycosylation are conserved.

A partial human OPG cDNA was cloned from a human 30 kidney cDNA library using the following rat-specific oligonucleotides:

- 5'-GTG AAG CTG TGC AAG AAC CTG ATG-3' (SEQ ID NO:19)  
5'-ATC AAA GGC AGG GCA TAC TTC CTG-3' (SEQ ID NO:20)
- This PCR product was sequenced and used to design 35 primers for amplifying the 3' end of the human cDNA using a human OPG genomic clone in lambda as template:  
5'-TCCGTAAGAACAGCCCAGTGACC-3' (SEQ ID NO:29)

5'-CAGATCCTGAAGCTGCTCAGTTG-3' (SEQ ID NO:21)

The amplified PCR product was sequenced, and together with the 5' end sequence, was used to design 5' and 3' human-specific primers useful for amplifying 5 the entire human OPG cDNA coding sequences:

5'-AGCGCGCCGCGGGGACCAATGAACAAGTTG-3' (SEQ ID NO:22)

5'-AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3' (SEQ ID NO:23)

The full-length human PCR product was sequenced, then directionally cloned into the plasmid vector 10 pRcCMV (Invitrogen) using Not I and Xba I. The resulting plasmid was named pRcCMV-human OPG. The nucleotide sequence of the cloned product was compared to the rat and mouse OPG cDNA sequences. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA 15 sequences are approximately 78-88% identical to the human OPG cDNA. The human OPG cDNA sequence also contained a 401 aa LORF, and it was compared to the rat and mouse protein sequences. The predicted human OPG is approximatlely 85% identical, and about 90% identical 20 to the rat and mouse proteins, respectively. Sequence alignment of rat, mouse and human proteins show that they have been highly conserved during evolution. The human protein is predicted to have a N-terminal signal peptide, and 5 potential sites of N-linked 25 glycosylation, 4 of which are conserved between the rat and mouse OPG.

The DNA and predicted amino acid sequence of mouse OPG is shown in Figure 9A and 9B (SEQ ID NO:122). The DNA and predicted amino acid sequence of human OPG is 30 shown in Figure 9C an 9D (SEQ ID NO:124). A comparison of the rat, mouse and human OPG amino acid sequences is shown in Figure 9E and 9F.

Isolation of additional human OPG cDNA clones revealed the presence of a G to C base change at 35 position 103 of the DNA sequence shown in Figure 9C. This nucleotide change results in substitution of an

asparagine for a lysine at position 3 of the amino acid sequence shown in Figure 9C. The remainder of the sequence in clones having this change was identical to that in Figure 9C and 9D.

5

#### EXAMPLE 6

##### OPG three-dimensional structure modelling

The amino-terminal portion of OPG has homology to the extracellular portion of all known members of the TNFR superfamily (Figure 1C). The most notable motif in this region of TNFR-related genes is an about 40 amino acid, cysteine-rich repeat sequence which folds into distinct structures (Banner *et al.* (1993), *Cell* 73: 431-45). This motif is usually displayed in four (range 3-6) tandem repeats (see Figure 1C), and is known to be involved in ligand binding (Beutler and van Huffel (1994), *Science* 264: 667-73). Each repeat usually contains six interspaced cysteine residues, which are involved in forming three intradomain disulfide bonds, termed SS1, SS2, and SS3 (Banner *et al.*, *ibid*). In some receptors, such as TNFR2, CD30 and CD40, some of the repeat domains contain only two intrachain disulfide bonds (SS1 and SS3).

The human OPG sequence was aligned to a TNFR1 extracellular domain profile using methods described by Luethy, *et al.*, *ibid*, and the results were graphically displayed using the PrettyPlot program from the Wisconsin Package, version 8.1 (Genetics Computer Group, Madison, WI) (Figure 10). The alignment indicates a clear conservation of cysteine residues involved in formation of domains 1-4. This alignment was then used to construct a three-dimensional (3-D) model of the human OPG N-terminal domain using the known 3-D structure of the extracellular domain of p55 TNFR1 (Banner *et al.*, *ibid*) as the template. To do this the atomic coordinates of the peptide backbone and side chains of identical residues were copied from the

crystal structure coordinates of TNFR1. Following this, the remaining coordinates for the insertions and different side chains were generated using the LOOK program (Molecular Applications Group, Palo Alto, CA).  
5 The 3-D model was then refined by minimizing its conformational energy using LOOK.

By analogy with other TNFR family members, it is assumed that OPG binds to a ligand. For the purpose of modelling the interaction of OPG with its ligand, the crystal structure of TNF- $\beta$  was used to simulate a 3-D representation of an "OPG ligand". This data was graphically displayed (see Figure 11) using Molscript (Kraulis (1991), J. Appl. Cryst. 24: 946-50). A model for the OPG/ligand complex with 3 TNF $\beta$  and 3 OPG molecules was constructed where the relative positions of OPG are identical to TNFR1 in the crystal structure. This model was then used to find the residues of OPG that could interact with its ligand using the following approach: The solvent accessible area of all residues in the complex and one single OPG model were calculated. The residues that have different accessibility in the complex than in the monomer are likely to interact with the ligand.  
10  
15  
20  
25

The human and mouse OPG amino acid sequences were realigned using this information to highlight sequences comprising each of the cysteine rich domains 1-4 (Figure 12A and 12B). Each domain has individual structural characteristics which can be predicted.  
30

Domain 1: Contains 4 cysteines involved in SS2 (C41 to C54) and SS3 (C44 to C62) disulfide bonds. Although no SS1 bond is evident based on disulfide bridges, the conserved tyrosine at position 28 is homologous to Y20 in TNFR1, which is known to be involved in interacting with H66 to aid in domain formation. OPG has a homologous histidine at position  
35

75, suggesting OPG Y28 and H75 stack together in the native protein, as do the homologous residues in TNFR1. Therefore, both of these residues may indeed be important for biological activity, and N-terminal OPG 5 truncations up to and beyond Y28 may have altered activity. In addition, residues E34 and K43 are predicted to interact with a bound ligand based on our 3-dimensional model.

10        Domain 2: Contains six cysteines and is predicted to contain SS1 (C65 to C80), SS2 (C83 to C98) and SS3 (C87 to C105) disulfide bonds. This region of OPG also contains an region stretching from P66-Q91 which aligns to the portion of TNFR1 domain 2 which forms close contacts with TNF $\beta$  (see above), and may interact with 15 an OPG ligand. In particular residues P66, H68, Y69, Y70, T71, D72, S73, H75, T76, S77, D78, E79, L81, Y82, P85, V86, K88, E89, L90, and Q91 are predicted to interact with a bound ligand based on our structural data.

20        Domain 3: Contains 4 cysteines involved in SS1 (C107 to C118) and SS3 (C124 to C142) disulfide bonds, but not an SS2 bond. Based on our structural data, residues E115, L118 and K119 are predicted in to interact with an OPG ligand.

25        Domain 4: Contains 4 cysteines involved in SS1 (C145 to C160) and SS3 (C166 to C185) disulfide bonds, but not an SS2 bond, similar to domain 3. Our structural data predict that E153 and S155 interact with an OPG ligand.

30        Thus, the predicted structural model for OPG identifies a number of highly conserved residues which are likely to be important for its biological activity.

**EXAMPLE 7**

Production of recombinant secreted  
OPG in mammalian cells

To determine if OPG is actually a secreted protein, mouse OPG cDNA was fused to the human IgG1 Fc domain as a tag (Capon *et al.* *Nature* 337, 525-531 (1989)), and expressed in human 293 fibroblasts. Fc fusions were carried out using the vector pFc-A3. pFc-A3 contains the region encoding the Fc portion of human immunoglobulin IgG- $\gamma$ 1 heavy chain (Ellison *et al.* *ibid*) from the first amino acid of the hinge domain (Glu-99) to the carboxyl terminus and is flanked by a 5'-NotI fusion site and 3'-SalI and XbaI sites. The plasmid was constructed by PCR amplification of the human spleen cDNA library (Clontech). PCR reactions were in a final volume of 100  $\mu$ l and employed 2 units of Vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10  $\mu$ M  $(\text{NH}_4)_2\text{SO}_4$ , 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100 with 400  $\mu$ M each dNTP and 1 ng of the cDNA library to be amplified together with 1  $\mu$ M of each primer. Reactions were initiated by denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 73°C for 2 min. The 5' primer  
5' ATAGCGCCGCTGAGCCCAAATCTTGTGACAAAACTCAC 3'  
(SEQ ID NO:24)  
incorporated a NotI site immediately 5' to the first residue (Glu-99) of the hinge domain of IgG- $\gamma$ 1. The 3' primer  
5' -TCTAGAGTCGACTTATCATTTACCCGGAGACAGGGAGAGGGCTCTT-3'  
(SEQ ID NO:25)  
incorporated SalI and XbaI sites. The 717-bp PCR product was digested with NotI and SalI, isolated by electrophoresis through 1% agarose (FMC Corp.), purified by the Geneclean procedure (BIO 101, Inc.) and cloned into NotI, SalI-digested pBluescript II KS vector (Stratagene). The insert in the resulting plasmid, pFc-A3, was sequenced to confirm the fidelity of the PCR reaction.

The cloned mouse cDNA in plasmid pRcCMV-MuOPG was amplified using the following two sets of primer pairs:

Pair 1:

5 5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAAG-3'  
(SEQ ID NO:26)  
5'-CCTCTGCAGGCCGCTAACGAGCTTATTCACGGATTGAACCTG-3'  
(SEQ ID NO:27)

Pair 2:

10 5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAAG-3'  
(SEQ ID NO:28)  
5'-CCTCTGCAGGCCGCTGGCATTTCTTCTG-3'  
(SEQ ID NO:30)

The first pair amplifies the entire OPG LORF, and creates a NotI restriction site which is compatible with the in-frame Not I site in Fc fusion vector pFcA3. pFcA3 was prepared by engineering a NotI restriction site 5' to aspartic acid residue 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the junction between the OPG and IgG Fc region. This product, when linked to the Fc portion, would encode all 401 OPG residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (Fl.Fc). The second primer pair amplifies the DNA sequences encoding the first 180 amino acid residues of OPG, which encompasses its putative ligand binding domain. As above, the 3' primer creates an artificial Not I restriction site which fuses the C-terminal truncated OPG LORF at position threonine 180 directly to the IgG1 Fc domain (CT.fc).

The amino acid sequence junction linking OPG residue 401 and aseptic acid residue 221 of the human Fc region can be modified as follows: The DNA encoding residues 216-220 of the human Fc region can be deleted as described below, or the cysteine residue corresponding to C220 of the human Fc region can be

5 mutated to either serine or alanine. OPF-Fc fusion protein encoded by these modified vectors can be transfected into human 293 cells, or CHO cells, and recombinant OPG-Fc fusion protein purified as described below.

Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is capable of episomal replication in 293-EBNA-1 cells.

10 The parent pCEP4, and pCEP4-F1.Fc and pCEP4-CT.Fc vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 µg/ml hygromycin to select for vector expression, and the resulting drug-

15 resistant mass cultures were grown to confluence. The cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced

20 by the drug resistant 293 cultures. In the pCEP4-F1.FC and the pCEP4-CT.FC conditioned media, unique bands of the predicted sizes were abundantly secreted (see Figures 13B and 13C). The full-length Fc fusion protein accumulated to a high concentration, indicating that it

25 may be stable. Both Fc fusion proteins were detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant OPG products.

The full length OPG-Fc fusion protein was purified

30 by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira *et al.* (*J. Biol. Chem.* 262, 10-35 (1987)).

The following amino acid sequence was read after 19 cycles:

NH<sub>2</sub>-E T L P P K Y L H Y D P E T G H Q L L-CO<sub>2</sub>H  
(SEQ ID NO:31)

5 This sequence was identical to the predicted mouse OPG amino acid sequence beginning at amino acid residue 22, suggesting that the natural mammalian leader cleavage site is between amino acid residues Q21-E22, not between Y31-D32 as originally predicted. The 10 expression experiments performed in 293-EBNA cells with pCEP4-F1.Fc and pCEP4-CT.Fc demonstrate that OPG is a secreted protein, and may act systemically to bind its ligand.

Procedures similar to those used to construct and 15 express the muOPG[22-180]-Fc and muOPG[22-401]-Fc fusions were employed for additional mouse and human OPG-Fc fusion proteins.

Murine OPG cDNA encoding amino acids 1-185 fused to the Fc region of human IgG1 [muOPG Ct(185).Fc] was 20 constructed as follows. Murine OPG cDNA from plasmid pRcCMV Mu Osteoprotegerin (described in Example 5) was amplified using the following primer pair in a polymerase chain reaction as described above:

1333-82:

25 5'-TCC CTT GCC CTG ACC ACT CTT-3'  
(SEQ ID NO:32)

1333-80:

5'-CCT CTG CGG CCG CAC ACA CGT TGT CAT GTG TTG C-3'  
(SEQ ID NO:33)

30 This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 63-185 (corresponding to bp 278-645) of the OPG reading frame as shown in Figure 9A. The 3' primer contains a Not I restriction site which is compatible with the in-frame 35 Not I site of the Fc fusion vector pFcA3. The product also spans a unique EcoRI restriction site located at

bp 436. The amplified PCR product was purified, cleaved with NotI and EcoRI, and the resulting EcoRI-NotI restriction fragment was purified. The vector pCEP4 having the murine 1-401 OPG-Fc fusion insert was 5 cleaved with EcoRI and NotI, purified, and ligated to the PCR product generated above. The resulting pCEP4-based expression vector encodes OPG residues 1-185 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-185.Fc fusion 10 vector was transfected into 293 cells, drug selected, and conditioned media was produced as described above. The resulting secreted murine OPG 1-185.Fc fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended 15 procedures.

Murine OPG DNA encoding amino acid residues 1-194 fused to the Fc region of human IgG1 (muOPG Ct(194).Fc) was constructed as follows. Mouse OPG cDNA from plasmid pRCCMV Mu-Osteoprotegerin was amplified using the 20 following primer pairs:

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3'  
(SEQ ID NO:34)

1333-81:

25 5'-CCT CTG CGG CCG CCT TTT GCG TGG CTT CTC TGT T-3'  
(SEQ ID NO:35)

This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 70-194 (corresponding to bp 298-672) of the OPG reading frame. 30 The 3' primer contains a Not I restriction site which is compatible with the in-frame Not I site of the Fc fusion vector pFca3. The product also spans a unique EcoRI restriction site located at bp 436. The amplified PCR product was cloned into the murine OPG[1-401] Fc 35 fusion vector as described above. The resulting pCEP4-based expression vector encodes OPG residues 1-194

directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-194.Fc fusion vector was transfected into 293 cells, drug selected, and conditioned media was produced. The resulting 5 secreted fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acids 1-401 fused to the Fc region of human IgG1 was constructed as follows. 10 Human OPG DNA in plasmid pRrCMV-hu osteoprotegerin (described in Example 5) was amplified using the following oligonucleotide primers:

1254-90:

5'CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'  
15 (SEQ ID NO:36)

1254-95:

5'-CCT CTG CGG CCG CTA AGC AGC TTA TTT TTA CTG AAT GG-3'  
(SEQ ID NO:37)

The resulting PCR product encodes the full-length 20 human OPG and creates a Not I restriction site which is compatible with the in-frame Not I site Fc fusion vector FcA3. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above. The resulting expression vector encodes human OPG residues 25 1-401 directly followed by 227 amino acid residues of the human IgG1 Fc region. Conditioned media from transfected and drug selected cells was produced and the huOPG F1.Fc fusion product was purified by Protein-A column chromatography (Pierce) using the 30 manufacturers recommended procedures.

Human OPG DNA encoding amino acid residues 1-201 fused to the Fc region of human IgG1 [huOPG Ct(201).Fc] was constructed as follows. The cloned human OPG cDNA from plasmid pRrCMV-hu osteoprotegerin was amplified by 35 PCR using the following oligonucleotide primer pair:

1254-90:

5'-CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'  
(SEQ ID NO:38)

1254-92:

5 5'-CCT CTG CGG CCG CCA GGG TAA CAT CTA TTC CAC-3'  
(SEQ ID NO:39)

This primer pair amplifies the human OPG cDNA region encoding amino acid residues 1-201 of the OPG reading frame, and creates a Not I restriction site at the 3' end which is compatible with the in-frame Not I site Fc fusion vector FcA3. This product, when linked to the Fc portion, encodes OPG residues 1-201 directly followed by all 221 amino acid residues of the human IgG1 Fc region. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above. Conditioned media from transfected and drug selected cells was produced, and the hu OPG Ct(201).Fc fusion products purified by Protein-A column chromatography (Pierce) using the manufacturer's recommended procedures.

20 The following procedures were used to construct and express unfused mouse and human OPG.

A plasmid for mammalian expression of full-length murine OPG (residues 1-401) was generated by PCR amplification of the murine OPG cDNA insert from pRcCMV Mu-Osteoprotegerin and subcloned into the expression vector pDSR $\alpha$  (DeClerck et. al. J. Biol. Chem. 266, 3893 (1991)). The following oligonucleotide primers were used:

1295-26:

30 5'-CCG AAG CTT CCA CCA TGA ACA AGT GGC TGT GCT GC-3'  
(SEQ ID NO:40)

1295-27:

5'-CCT CTG TCG ACT ATT ATA AGC AGC TTA TTT TCA CGG ATT G-3'  
(SEQ ID NO:41)

35 The murine OPG full length reading frame was amplified by PCR as described above. The PCR product

was purified and digested with restriction endonucleases Hind III and XbaI (Boehringer Mannheim, Indianapolis, IN) under the manufacturers recommended conditions, then ligated to Hind III and Xba I digested pDSR $\alpha$ . Recombinant clones were detected by restriction endonuclease digestion, then sequenced to ensure no mutations were produced during the PCR amplification steps.

The resulting plasmid, pDSR $\alpha$ -muOPG was introduced into Chinese hamster ovary (CHO) cells by calcium mediated transfection (Wigler *et al.* (1977), *Cell* 11: 233). Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the murine OPG recombinant protein was monitored by western blot analysis of CHO cell conditioned media. High expressing cells were selected, and OPG expression was further amplified by treatment with methotrexate as described (DeClerck *et al.*, *ibid.*). Conditioned media from CHO cell lines was produced for further purification of recombinant secreted murine OPG.

A plasmid for mammalian expression of full-length human OPG (amino acids 1-401) was generated by subcloning the cDNA insert in pRcCMV-hu Osteoprotegerin directly into vector pDSR $\alpha$  (DeClerck *et al.*, *ibid.*). The pRcCMV-OPG plasmid was digested to completion with Not I, blunt ended with Klenow, then digested to completion with XbaI. Vector DNA was digested with HindIII, blunt ended with Klenow, then digested with XbaI, then ligated to the OPG insert. Recombinant plasmids were then sequenced to confirm proper orientation of the human OPG cDNA.

The resulting plasmid pDSR $\alpha$ -huOPG was introduced into Chinese hamster ovary (CHO) cells as described

above. Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the human OPG recombinant protein was 5 monitored by western blot analysis of CHO cell conditioned media. High expressing clones were selected, and OPG expression was further amplified by treatment with methotrexate. Conditioned media from CHO cell lines expressing human OPG was produced for 10 protein purification.

Expression vectors for murine OPG encoding residues 1-185 were constructed as follows. Murine OPG cDNA from pRCCMV-Mu OPG was amplified using the following oligonucleotide primers:

15 1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:42)

1356-12:

5'-CCT CTG TCG ACT TAA CAC ACG TTG TCA TGT GTT GC-3'  
(SEQ ID NO:43)

20 This primer pair amplifies the murine OPG cDNA region encoding amino acids 63-185 of the OPG reading frame (bp 278-645) and contains an artificial stop codon directly after the cysteine codon (C185), which is followed by an artificial Sal I restriction 25 endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large 30 fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel 35 purified. This fragment, which contains an open reading frame encoding residues 1-185 was then subcloned into a

Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a cysteine residue located at position 185. Conditioned media from transfected and drug selected cells was produced as described above.

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:44)

1356-13:

10 5'-CCT CTG TCG ACT TAC TTT TGC GTG GCT TCT CTG TT-3'  
(SEQ ID NO:45)

This primer pair amplifies the murine OPG cDNA region encoding amino acids 70-194 of the OPG reading frame (bp 298-672) and contains an artificial stop codon directly after the lysine codon (K194), which is followed by an artificial Sal I restriction endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel purified. This fragment, which contains an open reading frame encoding residues 1-185 was then subcloned into a Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a lysine at position 194. Conditioned media from transfected and drug selected cells was produced as described above.

Several mutations were generated at the 5' end of the huOPG [22-401]-Fc gene that introduce either amino acid substitutions, or deletions, of OPG between

residues 22 through 32. All mutations were generated with the "QuickChange™ Site-Directed Mutagenesis Kit" (Stratagene, San Diego, CA) using the manufacturer's recommended conditions. Briefly, reaction mix  
5 containing huOPG [22-401]-Fc plasmid DNA template and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent E. coli  
10 XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to delete residues 22-26 of the human OPG gene, resulting in the production of a huOPG [27-401]-Fc fusion protein:

15 1436-11:

5'-TGG ACC ACC CAG AAG TAC CTT CAT TAT GAC-3' (SEQ ID NO:140)

1436-12:

5'-GTC ATA ATG AAG GTA CTT CTG GGT CCA-3' (SEQ ID NO:141)

20 The following primer pairs were used to delete residues 22-28 of the human OPG gene, resulting in the production of a huOPG [29-401]-Fc fusion protein:

1436-17:

5'-GGA CCA CCC AGC TTC ATT ATG ACG AAG AAA C-3' (SEQ ID NO:142)

1436-18:

25 5'-GTT TCT TCG TCA TAA TGA AGC TGG GTG GTC C-3' (SEQ ID NO:143)

The following primer pairs were used to delete residues 22-31 of the human OPG gene, resulting in the production of a huOPG [32-401]-Fc fusion protein:

1436-27:

30 5'-GTG GAC CAC CCA GGA CGA AGA AAC CTC TC-3' (SEQ ID NO:144)

1436-28:

5'-GAG AGG TTT CTT CGT CCT GGG TGG TCC AC-3' (SEQ ID NO:145)

35 The following primer pairs were used to change the codon for tyrosine residue 28 to phenylalanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc Y28F fusion protein:

1436-29:

5'-CGT TTC CTC CAA AGT TCC TTC ATT ATG AC-3' (SEQ ID NO:146)

1436-30:

5'-GTC ATA ATG AAG GAA CTT TGG AGG AAA CG-3' (SEQ ID NO:147)

5 The following primer pairs were used to change the codon for proline residue 26 to alanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc P26A fusion protein:

1429-83:

10 5'-GGA AAC GTT TCC TGC AAA GTA CCT TCA TTA TG-3 (SEQ ID NO:148)

1429-84:

5'-CAT AAT GAA GGT ACT TTG CAG GAA ACG TTT CC-3' (SEQ ID NO:149)

Each resulting muOPG [22-401]-Fc plasmid containing the appropriate mutation was then 15 transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in Example 11.

20

**EXAMPLE 8**

Expression of OPG in E. coli

A. Bacterial Expression Vectors

pAMG21

The expression plasmid pAMG21 can be derived from 25 the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be derived from the Amgen expression vector system described in US Patent No. 4,710,473. The pCFM1656 plasmid can be derived from the described pCFM836 plasmid (Patent No. 4,710,473) by: (a) 30 destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end ligation; (b) replacing the DNA sequence between the unique AatII and ClaI restriction sites containing the synthetic  $P_L$  promoter with a similar fragment 35 obtained from pCFM636 (patent No. 4,710,473) containing the  $P_L$  promoter

## AatII

5' CTAATTCCGCTCTCACCTACCAAACAATGCCCTGGCAAAAAATAAATTCTATAT-  
3' TGCAGATTAAGGCGAGAGTGGATGGTTACGGGGGACGTTTTATTAAAGTATA-

5 -AAAAAACATACAGATAACCATCTGGGTGATAAAATTATCTCTGGGGTGTTGACATAAA-  
-TTTTTGATGTCTATTGGTAGACGCCACTATTAATAGAGACCGCCACAACGTATTT-

-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)

-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

## 10 Clal

and then (c) substituting the small DNA sequence between the unique Clal and KpnI restriction sites with the following oligonucleotide:

5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACCGCGTTGGAATTGGTAC3'

15 (SEQ ID NO:48)

3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGAACCTTAAGC 5'

(SEQ ID NO:49)

## Clal KpnI

The expression plasmid pAMG21 can then be derived from pCFM1656 by making a series of site directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the BglII site (plasmid bp # 180) immediately 5' to the plasmid replication promoter PcopB and proceeding toward the plasmid replication genes, the base pair changes are as follows:

Table 4

	<u>pAMG21 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG21</u>
30	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	--	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
35	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
	# 1178	G/C	T/A
40	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A

	# 2480	A/T	T/A
	# 2499-2502	AGTG TCAC	GTCA CAGT
5	# 2642	TCCGAGC AGGCTCG	7 bp deletion
10	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[AatII sticky end] 5' GCGTAACGTATGCATGGTCTCC-  
(position #4358 in pAMG21) 3' TGCACGCATTGCATACTGACCGAGGG-  
20 -CCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAAGCAAAGGCTCAGTCGAAAGACT-  
-GGTACGCTCTCATCCCTGACGGTCCGTAGTTATTTGCTTCCGAGTCAGCTTCTGA-  
-GGGCCTTCGTTTATCTGTTGTTGTCGGTGAACGCTCTCTGAGTAGGACAATCCGC-  
25 -CCCGGAAAGCAAAATAGACAACAAACAGCCACTTGCAGAGGACTCATCCTGTTAGGCG-  
-CGGGAGCGGATTGAAACGTTGCGAAGCAACGGCCGGAGGGTGGCGGGCAGGACGCCGC-  
-GCCCTCGCCTAAACTTGCACGCTTCGTTGCCGGGCCCTCCACCGCCCGCCTGCAGGGCG-  
-CATAAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTGCGT-  
30 -GTATTTGACGGTCCGTAGTTAATTGCTTCCGGTAGGACTGCCTACCGAAAAACGCA-  
  
AatII  
-TTCTACAAACTCTTTGTTATTTCTAAATACATTCAAATATGGACGTCGTACTAAC-  
-AAGATGTTGAGAAAACAATAAAAGATTATGTAAGTTATACCTGCAGCATGAATTG-  
35 -TTTTAAAGTATGGCAATCAATTGCTCTGTTAAATTGCTTAGAAATACTTGGCAGC-  
-AAAATTTCATACCCGTTAGTTAACGAGGACAATTAAACGAAATCTTATGAAACCCTCG-  
-GGTTTGTGTATTGAGTTCATTTGCGCATTGGTTAAATGGAAAGTGACCGTGCCTTAC-  
40 -CCAAACACATAACTCAAAGTAAACGCGTAACCAATTACCTTCACTGGCACCGAATG-  
-TACAGCCTAATATTTGAAATATCCCAAGAGCTTTCTCGCATGCCACGCTAAC-  
-ATGTCGGATTATAAAACTTATAGGGTCTCGAAAAGGAAGCGTACGGGTGCGATTG-  
45 -ATTCTTTCTCTTTGGTTAAATCGTTGTTGATTATTGCTATATTATTTTC-  
-TAAGAAAAAGAGAAAACCAATTAGCAACAAACTAAATAAACGATATAAATAAAAG-  
-GATAATTATCAACTAGAGAAGGAACAATTATGGTATGTTCATACACGCTGTTAA-  
50 -CTATTAATAGTTGATCTCTTGTAAATTACCATACAAGTATGTCGTACATTTCAT-  
-AACTATCTATATAGTTGCTCTGAATGTGAAAACCTAACGATTCCGAAGCCATTAT-  
-TTGATAGATATCAACAGAAAGAGACTACACGTTGATTGTAAGGCTTCGGTAATA-  
-TAGCAGTATGAATAGGGAAACTAAACCCAGTGATAAGACCTGATGATTGCTTCTTAA-  
55 -ATCGTCATACTTATCCCTTGATTGGGTCACTATTCTGGACTACTAAAGCGAAGAAATT-  
-TTACATTGGAGATTTTATTACAGCATTGTTCAAATATATTCCAATTATCGGTG-  
-AATGTAACCTCTAAAAATAATGTCGTAAACAAAGTTATATAAGGTTAATTAGCCAC-  
60 -AATGATTGGAGTTAGAATAATCTACTATAGGATCATATTATTAAATTAGCGTCATCAT-

-TTACTAACCTCAATCTTATTAGATGATATCCTAGTATAAAATAATTAAATCGCAGTAGTA-
  
 -AATATTGCCTCCATTTTAGGGTAATTATCCAGAATTGAAATATCAGATTAAACCATAG-
 5 -TTATAACGGAGGTAAAAATCCCATTAAATAGGTCTTAACCTTATAGTCTAAATTGGTATC-
  
 -AATGAGGATAAATGATCGCGAGTAAATAATATTACAATGTACCATTAGTCATATCAG-
 -TTACTCCTATTACTAGCGCTCATTATTATAAGTGTACATGGTAAAATCAGTATAGTC-
  
 10 -ATAAGCATTGATTAATATCATATTGCTCTACAGGCTTAATTTATTAAATTATTCTGT-
 -TATTCGTAACTAAATTATACTAACGAAGATGTCCGAAATTAAATTAAAGACA-
  
 -AAGTGTGTCGGCATTTATGCTTCATACCCATCTCTTATCCTTACCTATTGTTGTC-
 -TTCACAGCAGCCGTAACAGAAAGTATGGGTAGAGAAATAGGAATGGATAACAAACAG-
 15 -GCAAGTTTGCGTGTATATATCATTAAAACGGTAATAGATTGACATTGATTCTAATAA-
 -CGTTCAAAACGCACAATATATAGTAATTGCCCCATTCTAACTGTAAACTAAGATTATT-
  
 -ATTGGATTTGTCACACTATTATATCGCTGAAATACAATTGTTAACATAAGTACCTG-
 -TAACCTAAAACAGTGTGATAATATAGCGAACCTTATGTTAACAAATTGTATTGAC-
 20 -TAGGATCGTACAGGTTACGCAAGAAAATGGTTGTTAGTCGATTAATCGATTGATT-
 -ATCCTAGCATGTCAAATGCGTTCTTTACCAACAAATATCAGCTAATTAGCTAAACTAA-
  
 25 -CTAGATTGTTTAACTAATTAAAGGAGGAATAACATATGGTTAACGCGTTGGAATTGCA-
 -GATCTAAACAAATTGATTAATTCCCTCTTATTGTATACCAATTGCGAACCTTAAGCT-
  
 SacII
 -GCTCACTAGTGTGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGGAAAGAA-
 -CGAGTGATCACAGCTGGACGTCGGCATGGTACCTTCGAATGAGCTCTAGGCGCCTTCTT-
 30 -GAAGAAGAAGAAGAAAGCCGAAAGGAAGCTGAGTTGGCTGCCACCGCTGAGCAATA-
 -CTTCTCTTCTTCTTCGGGCTTCCTCGACTCAACCGACGACGGTGGCGACTCGTTAT-
  
 35 -ACTAGCATAACCCCTTGGGGCTCTAAACGGGTCTGAGGGGTTTTGCTGAAAGGAGG-
 -TGATCGTATTGGGAACCCCGAGATTGCCAGAACTCCCCAAAAACGACTTCCCTC-
  
 -AACCGCTTTCACGCTTTACGC 3' [SacII sticky end] (SEQ ID NO:50)
 -TTGGCGAGAAGTGGCAGAAGTG 5' (position #5904 in pAMG21)
 (SEQ ID NO:46)

40 During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

45 pAMG22-His

The expression plasmid pAMG22-His can be derived from the Amgen expression vector pAMG22 by substituting the small DNA sequence between the unique NdeI (#4795) and EcoRI (#4818) restriction sites of pAMG22 with the 50 following oligonucleotide duplex:

NdeI                  NheI                  EcoRI  
 5' TATGAAACATCATCACCACCATCACCACATGCTAGCGTTAACCGCGTGG 3'

(SEQ ID NO:51)

3' ACTTTGTAGTAGTGGTAGTGGTAGTACGATCGCAATTGCGAACCTTAA 5'

(SEQ ID NO:52)

MetLysHisHisHisHisHisAlaSerValAsnAlaLeuGlu

(SEQ ID NO:168)

pAMG22

5       The expression plasmid pAMG22 can be derived from  
the Amgen expression vector pCFM1656 (ATCC #69576)  
which in turn be derived from the Amgen expression  
vector system described in US Patent No. 4,710,473  
granted December 1, 1987. The pCFM1656 plasmid can be  
10      derived from the described pCFM836 plasmid (Patent No.  
4,710,473) by: (a) destroying the two endogenous NdeI  
restriction sites by end filling with T4 polymerase  
enzyme followed by blunt end ligation; (b) replacing  
the DNA sequence between the unique AatII and ClaI  
15      restriction sites containing the synthetic PL promoter  
with a similar fragment obtained from pCFM636 (patent  
No. 4,710,473) containing the PL promoter

AatII

5'       CTAATTCCGCTCTCACCTACCAAACAATGCCCGGCTGCAAAAAAATAATTTCATAT-  
20      3' TGCAGATTAAGGCGAGAGTGGATGGTTGTTACGGGGGGACGTTTTATTAAAGTATA-

-AAAAAACATACAGATAACCCTGCGGTGATAAAATTATCTCTGGCGGTGTTGACATAAA-  
-TTTTTGATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAAGTGTATTT-

25      -TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)  
-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

ClaI

and then (c) substituting the small DNA sequence  
between the unique ClaI and KpnI restriction sites with  
30      the following oligonucleotide:

5' CGATTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTGGTAC 3'  
(SEQ ID NO:55)

3' TAAACTAAGATCTCCTCCTTATTGTATACCAATTGCGCACCTTAAGC 5'  
(SEQ ID NO:56)

35      ClaI

KpnI

The expression plasmid pAMG22 can then be derived from pCFM1656 by making a series of site directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the BglII site (plasmid bp # 180) immediately 5' to the plasmid replication promoter PcopB and proceeding toward the plasmid replication genes, the base pair changes are as follows:

Table 5

	<u>pAMG22 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG22</u>
10	# 204	T/A	C/G
	# 428	A/T	G/C
15	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
20	# 980	T/A	C/G
	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
25	# 1047	C/G	T/A
	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
30	# 2480	A/T	T/A
	# 2499-2502	AGTG TCAC	GTCA CAGT
35	# 2642	TCCGAGC AGGCTCG	7 bp deletion
	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

40 The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[AatII sticky end] (position #4358 in pAMG22)

5' GCGTAACGTATGCATGGTCTCCCCATGCGAGAGTAGGAACTGCCAGGCATCAA-  
 3' TGCACGCATTGCATACGTACCAAGAGGGTAGCCTCATCCCTGACGGTCCGTAGTT-  
  
 5 -ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTCGTTTATCTGTTGTTGTCGGTG-  
 -TATTTGCTTCCGAGTCAGCTTGACCCGAAAGCAAATAGACAACAAACAGCCAC-  
  
 10 -AACGCTCTCCTGAGTAGGACAAATCCGCCGGAGCGGAGTTGAACGTTGCGAAGCAACGG-  
 -TTGCGAGAGGACTCATCCTGTTAGGCGGCCCTCGCTAAACTGCAACGCTTCGTTGCC-  
  
 15 -CCCGGAGGGTGGCGGGCAGGACGCCCATAAACTGCCAGGCATCAAATTAAGCAGAAG-  
 -GGGCCTCCCACGCCGCTCGCGGGCGTATTGACGGTCCGTAGTTAATCGCTTC-  
  
 15 -GCCATCCTGACGGATGGCCTTTGCGTTCTACAAAATCTTTGTTATTTCTAAAT-  
 -CGGTAGGACTGCCTACCGAAAAACGCAAAGATGTTGAGAAAACAATAAAAGATTAA-  
  
 AatII  
 -ACATTCAAATATGGACGTCTCATATTAAATTTAAAAAATTCAATTGACAAATGCTAAATTC-  
 -TGTAAAGTTATACCTGCAGAGTATTAAAAATTAAAGTAAACTGTTACGATTAAAG-  
  
 20 -TTGATTAATATTCTCAATTGTGAGCGCTCACAAATTATCGATTGATTCTAGATTGTTT-  
 -AACTAATTATAAGAGTTAACACTCGCGAGTGTAAATAGCTAAACTAAGATCTAAACTCA-  
  
 25 -TAACTAATTAAAGGAGGAATAACATATGGTTAACCGGTTGGAATTGAGCTCACTAGTGT-  
 -ATTGATTAATTCCCTTATTGTATACCAATTGCGCAACCTTAAGCTCGAGTGATCACA-  
  
 SacII  
 -CGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCCGGAAAGAAGAAGAAGAAGAA-  
 -GCTGGACGTCCCATGGTACCTCGAATGAGCTCCTAGGCGCTTTCTTCTTCTTCTT-  
  
 30 -GAAAGCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC-  
 -CTTCGGGCTTCCTCGACTCAACCGACGACGGTGGCGACTCGTTATTGATCGTATTGG-  
  
 35 -CCTTGGGCCTCTAAACGGGCTTGTGAGGGGTTTTGCTGAAAGGAGGAACCGCTCTCA-  
 -GGAACCCCGAGATTGCCCAGAAACTCCCCAAAAACGACTTCCCTGGCGAGAAGT-  
  
 -CGCTCTCACGC 3' (SEQ ID NO:58)  
 -GCGAGAAGTG 5' (SEQ ID NO:57)  
  
 40 [SacII sticky end] (position #5024 in pAMG22)  
 During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.  
  
 45 B. Human OPG Met[32-401]  
 In the example, the expression vector used was pAMG21, a derivative of pCFM1656 (ATCC accession no. 69576) which contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter. 50 (See U.S. Patent No. 5,169,318 for description of the lux expression system). The host cell used was GM120 (ATCC accession no. 55764). This host has the lacIQ promoter and lacI gene integrated into a second site in

the host chromosome of a prototrophic E. coli K12 host. Other commonly used E. coli expression vectors and host cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human OPG polypeptide was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as a template plasmid pRcCMV-Hu OPG DNA containing the human OPG cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for 30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22 were phosphorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and KpnI sticky ends. The phosphorylated linker duplex formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and KpnI cohesive ends (see Figure 14A) and the KpnI and BamHI digested and purified PCR product generated using oligo primers #1257-20 and #1257-19 (see above) was directionally inserted between two sites of the plasmid vector pAMG21, namely the NdeI site and BamHI site, using standard recombinant DNA methodology (see Figure 14A and sequences below). The synthetic linker utilized E. coli codons and provided for a N-terminal methionine.

Two clones were selected and plasmid DNA isolated, and the human OPG insert was subsequently DNA sequence confirmed. The resulting pAMG21 plasmid containing

amino acids 32-401 of the human OPG polypeptide immediately preceded in frame by a methionine is referred to as pAMG21-huOPG met[32-401] or pAMG21-huOPG met[32-401].

5           Oligo#1257-19:

5'-TACGCACTGGATCCTTATAAGCAGCTTATTCTACTGATTGGAC-3'  
(SEQ ID NO:59)

             Oligo#1257-20:

5'-GTCCTCCTGGTACCTACCTAAAACAAC-3' (SEQ ID NO:60)

10          Oligo#1257-21:

5'-TATGGATGAAGAAACTTCTCATCAGCTGCTGTGATAATGTCCGCCGGTAC -3'  
(SEQ ID NO:61)

             Oligo#1257-22:

5'-CCGGCGGACATTATCACACACAGCAGCTGATGAGAAGTTCTTCATCCA-3'

15          (SEQ ID NO:47)

Cultures of pAMG21-huOPG met[32-401] in E. coli GM120 in 2XYT media containing 20 µg/ml kanamycin were incubated at 30°C prior to induction. Induction of huOPG met[32-401] gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and cultures were incubated at either 30°C or 37°C for a further 6 hours. After 6 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies and were then pelleted by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant huOPG met[32-401] gene product was produced insolubly in E. coli.

Some bacterial pellets were resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemlli sample buffer to 1X final, and β-mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE. A substantially more intense coomassie stained band of approximately 42kDa was observed on a

SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture (Figure 14B). The expected gene product would be 370 amino acids in 5 length and have an expected molecular weight of about 42.2 kDa.

Following induction at 37 °C for 6 hours, an additional culture was pelleted and either processed for isolation of inclusion bodies (see below) or 10 processed by microfluidizing. The pellet processed for microfluidizing was resuspended in 25mM Tris-HCl/pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and collected. An aliquot was removed of the collected 15 sample (microfluidized total lysate), and the remainder was pelleted at 20,000 x g for 20 minutes. The supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea 20 solution (microfluidized insoluble fraction). To an aliquot of either the total soluble, or insoluble fraction was added to an equal volume of 2X Laemmlli sample buffer and β-mercaptoethanol to 5% final concentration. The samples were then analyzed by 25 SDS-PAGE. A significant amount of recombinant huOPG met[32-401] gene product appeared to be found in the insoluble fraction.

To purify the recombinant protein, inclusion bodies were purified as follows: Bacterial cells were 30 separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water. This 35 suspension was transferred to a stainless steel cup

cooled in ice and subjected to sonic disruption using a Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts). The sonicated cell suspension was centrifuged in a Beckman Optima TLX 5 ultracentrifuge equipped with a TLA 100.3 rotor at 195,000 x g for 5 to 10 minutes at 23°C. The supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle. The pellets were collected by scraping with a micro spatula and 10 transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended. The volume was increased to 19.5 ml by the addition of 15 Percoll solution, mixed, and distributed into 3 Beckman Quick-Seal tubes (13 x 32 mm). Tubes were sealed according to manufacturers instructions. The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600 x g), 30 minutes. The tubes were examined for 20 the appropriate banding pattern. To recover the refractile bodies, gradient fractions were recovered and pooled, then diluted with water. The inclusion bodies were pelleted by centrifugation, and the protein concentration estimated following SDS-PAGE.

25 An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemlli sample buffer with 5%  $\beta$ -mercaptoethanol and resolved on a SDS-PAGE gel and the isolated inclusion bodies provide a highly purified recombinant huOPG[32-401] gene product. 30 The major ~42 kDa band observed after resolving inclusion bodies on a SDS-polyacrylamide gel was excised from a separate gel and the N-terminal amino acid sequence determined essentially as described (Matsudaira *et al.* J. Biol. Chem. 262, 10-35 (1987)). 35 The following sequence was determined after 19 cycles:

NH<sub>2</sub> -MDEETSHQLLCDKCPPGTY-COOH (SEQ ID NO:62)

This sequence was found to be identical to the first 19 amino acids encoded by the pAMG21 Hu-OPG met[32-401] expression vector, produced by a methionine residue  
5 provided by the bacterial expression vector.

C. Human OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. Isolated plasmid DNA of pAMG21-huOPG met[32-401] (see Section B) was cleaved with KpnI and BamHI restriction endonucleases and the resulting fragments were resolved on an agarose gel. The B fragment (about 1064 bp fragment) was isolated from the gel using standard methodology. Synthetic oligonucleotides (oligos) #1267-06 and #1267-07 were phosphorylated individually and allowed to form an oligo linker duplex, which contained NdeI and KpnI cohesive ends, using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated oligo linker containing NdeI and KpnI cohesive ends and the isolated about 1064 bp fragment of pAMG21-huOPG met[32-401] digested with KpnI and BamHI restriction endonucleases were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard recombinant DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] gene.

## Oligo #1267-06:

5'-TAT GGA AAC TTT TCC TCC AAA ATA TCT TCA TTA TGA TGA AGA AAC TTC  
30 TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC GGG TAC-3'  
(SEQ ID NO:63)

## Oligo #1267-07:

5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAG AAG TTT CTT CAT  
CAT AAT GAA GAT ATT TTG GAG GAA AAG TTT CCA-3'  
35 (SEQ ID NO:64)

Cultures of pAMG21-huOPG-met[22-401] in E. coli host 393 were placed in 2XYT media containing 20 µg/ml kanamycin and were incubated at 30°C prior to induction. Induction of recombinant gene product expression from the luxPR promoter of vector pAMG21 was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and incubation at either 30°C or 37°C for a further 6 hours. After 6 hours, bacterial cultures were pelleted by centrifugation (=30°C I+6 or 37°C I+6). Bacterial cultures were also either pelleted just prior to induction (=30°C PreI) or alternatively no autoinducer was added to a separate culture which was allowed to incubate at 30°C for a further 6 hours to give an uninduced (UI) culture (=30°C UI). Bacterial pellets of either 30°C PreI, 30°C UI, 30°C I+6, or 37°C I+6 cultures were resuspended, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Section B. Polyacrylamide gels were either stained with coomassie blue and/or Western transferred to nitrocellulose and immunoprobed with rabbit anti-mu OPG-Fc polyclonal antibody as described in Example 10. The level of gene product following induction compared to either an uninduced (30°C UI) or pre-induction (30°C PreI) sample.

D. Murine OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of the murine (mu) OPG (OPG) polypeptide was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1257-16 and #1257-15 as primers, plasmid pRCCMV-Mu OPG DNA as a template and thermocycling conditions as described in Section B. The PCR product was purified and cleaved with KpnI and

BamHI restriction endonucleases as described in Section B. Synthetic oligos #1260-61 and #1260-82 were phosphorylated individually and allowed to form an oligo linker duplex with NdeI and KpnI cohesive ends 5 using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1260-61 and #1260-82 containing NdeI and KpnI cohesive ends and the KpnI and 10 BamHI digested and purified PCR product generated using oligo primers #1257-16 and #1257-15 were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation 15 utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG met[22-401] gene.

Expression of recombinant muOPG met[22-401] 20 polypeptide from cultures of 393 cells harboring plasmid pAMG21-MuOPG met[22-401] following induction was determined using methods described in Section C.

Oligo #1257-15:

5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTC ACG GAT TGA AC-3'  
25 (SEQ ID NO:65)

Oligo #1257-16:

5'-GTG CTC CTG GTA CCT ACC TAA AAC AGC ACT GCA CAG TG-3'  
(SEQ ID NO:66)

Oligo #1260-61:

30 5'-TAT GGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA TCC GGA AAC TGG  
TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC GGG TAC-3'  
(SEQ ID NO:67)

Oligo #1260-82:

35 5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CCG GAT CGT  
AAT GCA GGT ATT TTG GAG GCA GAG TTT CCA-3'  
(SEQ ID NO:68)

E. Murine OPG met[32-401]

A DNA sequence coding for an N-terminal methionine and amino acids 32 through 401 of murine OPG was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. To accomplish this, Synthetic oligos #1267-08 and #1267-09 were phosphorylated individually and allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1267-08 and #1267-09 containing NdeI and KpnI cohesive ends, and the KpnI and BamHI digested and purified PCR product described earlier (see Section D), was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[32-401] gene.

Expression of recombinant muOPG-met [32-401] polypeptide from cultures of 393 cells harboring the pAMG21 recombinant plasmid following induction was determined using methods described in Section C.

Oligo #1267-08:

5'-TAT GGA CCC AGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC  
GGG TAC-3' (SEQ ID NO:69)

Oligo #1267-09:

30 5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CTG GGT  
CCA-3' (SEQ ID NO:70)

F. Murine OPG met-lys[22-401]

A DNA sequence coding for an N-terminal methionine followed by a lysine residue and amino acids 22 through 401 of murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as

follows. Synthetic oligos #1282-95 and #1282-96 were phosphorylated individually and allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1282-95 and #1282-96 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product described in Section D was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG-Met-Lys[22-401] gene.

Expression of recombinant MuOPG Met-Lys[22-401] polypeptide from transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1282-95:

5'-TAT GAA AGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA TCC GGA AAC  
TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC GGG TAC-3'  
(SEQ ID NO:71)

Oligo #1282-96:

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CCG GAT CGT  
AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT TCA-3' (SEQ ID NO:72)  
G. Murine OPG met-lys-(his)<sub>7</sub>[22-401]

A DNA sequence coding for N-terminal residues Met-Lys-His-His-His-His-His (=MKH) followed by amino acids 22 through 401 of Murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-50 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section

B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases and purified. The NdeI and BamHI digested and purified PCR 5 product generated using oligo primers #1300-50 and #1257-15 was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard DNA methodology. The ligation mixture was transformed into 10 *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing performed to verify the DNA sequence of the muOPG-MKH[22-401] gene.

Expression of recombinant MuOPG-MKH[22-401] polypeptide from transformed 393 cultures harboring the 15 recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1300-50:

5'-GTT CTC CTC ATA TGA AAC ATC ATC ACC ACC ATC ATG AAA CTC TGC  
CTC CAA AAT ACC TGC ATT ACG AT-3' (SEQ ID NO:73)

20 Oligo #1257-15: see Section D

H. Murine OPG met-lys[22-401](his)7

A DNA sequence coding for a N-terminal met-lys, amino acids 22 through 401 murine OPG, and seven histidine residues following amino acid 401 (=muOPG 25 MK[22-401]-H7), was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-49 and #1300-51 as primers and pAMG21-muOPG met[22-401] DNA as template. Thermocycling conditions 30 were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was 35 directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation was

transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG  
5 MK[22-401]-H7 gene.

Expression of the recombinant muOPG MK-[22-401]-H7 polypeptide from a transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

10 Oligo #1300-49:

5'-GTT CTC CTC ATA TGA AAG AAA CTC TGC CTC CAA AAT ACC TGC A-3'  
(SEQ ID NO:74)

Oligo #1300-51:

5'-TAC GCA CTG GAT CCT TAA TGA TGG TGA TGG TGA TGA TGT AAG CAG CTT  
15 ATT TTC ACG GAT TGA ACC TGA TTC CCT A-3' (SEQ ID NO:75)

I. Murine OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-74 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[27-401] gene.

35 Expression of recombinant muOPG-met[27-401] polypeptide from a transfected 393 culture harboring

the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo#1309-74:

5' -GTT CTC CTC ATA TGA AAT ACC TGC ATT ACG ATC CGG AAA CTG GTC AT-  
5 3' (SEQ ID NO:76)

Oligo#1257-15: See Section D

J. Human OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of human OPG was placed  
10 under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-75 and #1309-76 as primers and plasmid pAMG21-huOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section  
15 B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with AseI and BamHI restriction endonucleases, and purified. The AseI and BamHI digested and purified PCR product above was directionally inserted between the  
20 NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into  
E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to  
25 verify the DNA sequence of the huOPG-met[27-401] gene.

Expression of the recombinant huOPG-met[27-401] polypeptide following induction of from transfected 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

30 Oligo #1309-75:

5' -GTT CTC CTA TTA ATG AAA TAT CTT CAT TAT GAT GAA GAA ACT T-3'  
(SEQ ID NO:77)

Oligo #1309-76:

35 5' -TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTT ACT GAT T-3'  
(SEQ ID NO:78)

K. Murine OPG met[22-180]

A DNA sequence coding for a N-terminal methionine and amino acids 22 through 180 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-72 and #1309-73 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[22-180] gene.

Expression of recombinant muOPG-met[22-180] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1309-72:

25 5'-GTT CTC CTC ATA TGG AAA CTC TGC CTC CAA AAT ACC TGC A-3'  
(SEQ ID NO:79)

Oligo #1309-73:

5'-TAC GCA CTG GAT CCT TAT GTT GCA TTT CCT TTC TGA ATT AGC A-3'  
(SEQ ID NO:80)

30 L. Murine OPG met[27-180]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 180 of murine OPG was placed under the control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed 35 using oligonucleotides #1309-74 (see Section I) and #1309-73 (see Section K) as primers and plasmid pAMG21-

muOPG met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG met[27-180] gene.

Expression of recombinant muOPG met[27-180] polypeptide from cultures of transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

M. Murine OPG met[22-189] and met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 189, or 22 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1337-92 and #1337-93 (=muOPG-189 linker) or #1333-57 and #1333-58 (=muOPG-194 linker) were phosphorylated individually and allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-muOPG-met[22-401] was cleaved with KpnI and BspEI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~413 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1337-92 and #1337-93 (muOPG-189 linker) or oligos #1333-57 and #1333-58 (muOPG-194 linker) containing BspEI and BamHI

cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites 5 of pAMG21-muOPG met[22-401] using standard methodology. Each ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify 10 the DNA sequence of either the muOPG-met[22-189] or muOPG-met[22-194] genes.

Expression of recombinant muOPG-met[22-189] and muOPG-met[22-194] polypeptides from recombinant pAMG21 plasmids transformed into 393 cells was determined 15 using methods described in Section C.

Oligo #1337-92:

5'-CCG GAA ACA GAT AAT GAG-3' (SEQ ID NO:81)

Oligo #1337-93:

5'-GAT CCT CAT TAT CTG TTT-3' (SEQ ID NO:82)

20 Oligo #1333-57:

5'-CCG GAA ACA GAG AAG CCA CGC AAA AGT AAG-3'

(SEQ ID NO:83)

Oligo #1333-58:

5'-GAT CCT TAC TTT TGC GTG GCT TCT CTG TTT-3'

25 (SEQ ID NO:84)

N. Murine OPG met[27-189] and met[27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 189, or 27 through 194 of murine OPG was placed under control of the lux 30 PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers either "muOPG-189 linker" or "muOPG-194 linker" (see Section M) containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG- 35 met[22-401] digested with KpnI and BspEI restriction endonucleases were directionally inserted between the

KpnI and BamHI sites of plasmid pAMG21-muOPG-met[27-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were 5 selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the muOPG met[27-189] or muOPG met[27-194] genes.

Expression of recombinant muOPG met[27-189] and muOPG met[27-194] following induction of 393 cells 10 harboring recombinant pAMG21 plasmids was determined using methods described in Section C.

O. Human OPG met[22-185], met[22-189], met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 185, 22 through 189, 15 or 22 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1331-87 and #1331-88 (=huOPG-185 linker), #1331-89 and #1331-90 (=huOPG- 20 189 linker), or #1331-91 & #1331-92 (=huOPG-194 linker) were phosphorylated individually and each allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-huOPG-met[27-401] was restricted with KpnI and NdeI 25 restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~407 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1331-87 and #1331-88 30 (huOPG-185 linker), oligos #1331-89 and #1331-90 (huOPG-189 linker), or oligos #1331-91 and #1331-92 (huOPG-194 linker) [each linker contains NdeI and BamHI cohesive ends], and the isolated ~407 bp B fragment of 35 plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of plasmid

pAMG21-huOPG-met[22-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the huOPG-met[22-185], huOPG-met[22-189], or huOPG-met[22-194] genes.

5 Expression of recombinant huOPG-met[22-185], huOPG-met[22-189] or huOPG-met[22-194] in transformed 10 393 cells harboring recombinant pAMG21 plasmids following induction was determined using methods described in Section C.

Oligo #1331-87:

5'-TAT GTT AAT GAG-3' (SEQ ID NO:85)

15 Oligo #1331-88:

5'-GAT CCT CAT TAA CA-3' (SEQ ID NO:86)

Oligo #1331-89:

5'-TAT GTT CCG GAA ACA GTT AAG-3' (SEQ ID NO:87)

Oligo #1331-90:

20 5'-GAT CCT TAA CTG TTT CCG GAA CA-3' (SEQ ID NO:88)

Oligo #1331-91:

5'-TAT GTT CCG GAA ACA GTG AAT CAA CTC AAA AAT AAG-3'

(SEQ ID NO:89)

Oligo #1331-92:

25 5'-GAT CCT TAT TTT TGA GTT GAT TCA CTG TTT CCG GAA CA-3' (SEQ ID NO:90)

P. Human OPG met[27-185], met[27-189], met [27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 185, 27 through 189, 30 or 27 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers "huOPG-185 linker", "huOPG-189 linker", or "huOPG-194 linker" (See Section 35 O) each containing NdeI and BamHI cohesive ends, and the isolated ~407 bp B fragment of plasmid pAMG21-

huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases (See Section O) were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[27-401] (See Section J) 5 using standard methodology. Each ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA isolated, and DNA sequencing performed to verify the DNA sequence of either the 10 huOPG-met[27-185], huOPG-met[27-189], or huOPG-met[27-194] genes.

Expression of recombinant huOPG-met[27-185], huOPG-met[27-189], and huOPG-met[27-194] from recombinant pAMG21 plasmids transformed into 393 cells 15 was determined using methods described in Section C.

O. Murine OPG met[27-401] (P33E, G36S, A45P)

A DNA sequence coding for an N-terminal methionine and amino acids 27 through 48 of human OPG followed by amino acid residues 49 through 401 of murine OPG was 20 placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows.

Purified plasmid DNA of pAMG21-huOPG-met[27-401] (See Section J) was cleaved with AatII and KpnI restriction endonucleases and a ~1075 bp B fragment isolated from 25 an agarose gel using standard recombinant DNA methodology. Additionally, plasmid pAMG21-muOPG-met[22-401] DNA (See Section D) was digested with KpnI and BamHI restriction endonucleases and the ~1064 bp B fragment isolated as described above. The isolated 30 ~1075 bp pAMG21-huOPG-met[27-401] restriction fragment containing AatII & KpnI cohesive ends (see above), the ~1064 bp pAMG21-muOPG-met[22-401] restriction fragment containing KpnI and BamHI sticky ends and a ~5043 bp restriction fragment containing AatII and BamHI 35 cohesive ends and corresponding to the nucleic acid sequence of pAMG21 between AatII & BamHI were ligated

using standard recombinant DNA methodology. The ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, and the presence of the 5 recombinant insert in the plasmid verified using standard DNA methodology. muOPG-27-401 (P33E, G36S, A45P) gene. Amino acid changes in muOPG from proline-33 to glutamic acid-33, glycine-36 to serine-36, and alanine-45 to proline-45, result from replacement of 10 muOPG residues 27 through 48 with huOPG residues 27 through 48.

Expression of recombinant muOPG-met[27-401] (P33E, G36S, A45P) from transformed 393 cells harboring the recombinant pAMG21 plasmid was determined using methods 15 described in Section C.

R. Murine OPG met-lys-(his)<sub>7</sub>-ala-ser-(asp)<sub>4</sub>-lys[22-401]  
(A45T)

A DNA sequence coding for an N-terminal His tag and enterokinase recognition sequence which is (NH<sub>2</sub> to 20 COOH terminus): Met-Lys-His-His-His-His-His-Ala-Ser-Asp-Asp-Asp-Lys (=HEK), followed by amino acids 22 through 401 of the murine OPG polypeptide was placed under control of the lac repressor regulated Ps4 promoter as follows. pAMG22-His (See Section A) was 25 digested with NheI and BamHI restriction endonucleases, and the large fragment (the A fragment) isolated from an agarose gel using standard recombinant DNA methodology. Oligonucleotides #1282-91 and #1282-92 were phosphorylated individually and allowed to form an 30 oligo linker duplex using methods previously described (See Section B). The phosphorylated linker duplex formed between oligos #1282-91 and #1282-92 containing NheI and KpnI cohesive ends, the KpnI and BamHI digested and purified PCR product described (see 35 Section D), and the A fragment of vector pAMG22-His

digested with NheI and BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into E. coli host GM120 by electroporation utilizing the manufacturer's protocol. Clones were 5 selected, plasmid DNA isolated and DNA sequencing performed to verify the DNA sequence of the muOPG- HEK[22-401] gene. DNA sequencing revealed a spurious mutation in the natural muOPG sequence that resulted in a single amino acid change of Alanine-45 of muOPG 10 polypeptide to a Threonine.

Expression of recombinant muOPG-HEK[22-401] (A45T) from GM120 cells harboring the recombinant pAMG21 plasmid was determined using methods similar to those described in Section C, except instead of addition of 15 the synthetic autoinducer, IPTG was added to 0.4 mM final to achieve induction.

Oligo #1282-91:

5'-CTA GCG ACG ACG ACG ACA AAG AAA CTC TGC CTC CAA AAT ACC TGC ATT  
ACG ATC CGG AAA CTG GTC ATC AGC TGC TGT GTG ATA AAT GTG CTC CGG  
20 GTA C-3' (SEQ ID NO:91)

Oligo #1282-92:

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CCG GAT CGT  
AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT TGT CGT CGT CGT CG-3'  
(SEQ ID NO:92)

25 S. Human OPG met-arg-gly-ser-(his)<sub>6</sub>[22-401]

Eight oligonucleotides (1338-09 to 1338-16 shown below) were designed to produce a 175 base fragment as overlapping, double stranded DNA. The oligos were annealed, ligated, and the 5' and 3' oligos were used 30 as PCR primers to produce large quantities of the 175 base fragment. The final PCR gene products were digested with restriction endonucleases ClaI and KpnI to yield a fragment which replaces the N-terminal 28 codons of human OPG. The ClaI and KpnI digested PCR 35 product was inserted into pAMG21-huOPG [27-401] which had also been cleaved with ClaI and KpnI. Ligated DNA

was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence.

5 Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Expression of huOPG Met-Arg-

10 Gly-Ser-(His)<sub>6</sub> [22-401] resulting in the formation of large inclusion bodies and the protein was localized to the insoluble (pellet) fraction.

1338-09:

ACA AAC ACA ATC GAT TTG ATA CTA GA (SEQ ID NO:93)

15 1338-10:

TTT GTT TTA ACT AAT TAA AGG AGG AAT AAA ATA TGA GAG GAT CGC ATC AC  
(SEQ ID NO:94)

1338-11:

CAT CAC CAT CAC GAA ACC TTC CCG CCG AAA TAC CTG CAC TAC GAC GAA GA  
20 (SEQ ID NO:95)

1338-12:

AAC CTC CCA CCA GCT GCT GTG CGA CAA ATG CCC GCC GGG TAC CCA AAC A  
(SEQ ID NO:96)

1338-13:

25 TGT TTG GGT ACC CGG CGG GCA TTT GT (SEQ ID NO:97)

1338-14:

CGC ACA GCA GCT GGT GGG AGG TTT CTT CGT CGT AGT GCA GGT ATT TCG GC  
(SEQ ID NO:98)

1338-15:

30 GGG AAG GTT TCG TGA TGG TGA TGG TGA TGC GAT CCT CTC ATA TTT TAT T  
(SEQ ID NO:99)

1338-16:

CCT CCT TTA ATT AGT TAA AAC AAA TCT AGT ATC AAA TCG ATT GTG TTT GT  
(SEQ ID NO:100)

35 T. Human OPG met-lys[22-401] and met(lys)3[22-401]

To construct the met-lys and met-(lys)3 versions of human OPG[22-401], overlapping oligonucleotides were designed to add the appropriate number of lysine

residues. The two oligos for each construct were designed to overlap, allowing two rounds of PCR to produce the final product. The template for the first PCR reaction was a plasmid DNA preparation containing 5 the human OPG 22-401 gene. The first PCR added the lysine residue(s). The second PCR used the product of the first round and added sequence back to the first restriction site, ClaI.

The final PCR gene products were digested with 10 restriction endonucleases ClaI and KpnI, which replace the N-terminal 28 codons of hu OPG, and then ligated into plasmid pAMG21-hu OPG [27-401] which had been also digested with the two restriction endonucleases.

Ligated DNA was transformed into competent host cells 15 of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell 20 lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Neither construct had a detectable level of protein expression and inclusion bodies were not visible. The DNA 25 sequences were confirmed by DNA sequencing.

Oligonucleotide primers to prepare Met-Lys huOPG[22-401]:

1338-17:

ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT AAA GGA  
30 GGA ATA AAA TG (SEQ ID NO:101)

1338-18:

CTA ATT AAA GGA GGA ATA AAA TGA AAG AAA CTT TTC CTC CAA AAT ATC  
(SEQ ID NO:102)

1338-20:

35 TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID NO:103)

Oligonucleotide primers to prepare Met-(Lys)<sub>3</sub>-huOPG[22-401]:

1338-17:

ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT AAA GGA  
5 GGA ATA AAA TG (SEQ ID NO:104)

1338-19:

CTA ATT AAA GGA GGA ATA AAA TGA AAA AAA AAG AAA CTT TTC CTC CAA  
AAT ATC (SEQ ID NO:105)

1338-20:

10 TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID NO:106)

U. Human and Murine OPG [22-401]/Fc Fusions

Four OPG-Fc fusions were constructed where the Fc region of human IgG1 was fused at the N-terminus of either human or murine Osteoprotegerin amino acids 22 15 to 401 (referred to as Fc/OPG [22-401]) or at the C-terminus (referred to as OPG[22-401]/Fc). Fc fusions were constructed using the fusion vector pFc-A3 described in Example 7.

All fusion genes were constructed using standard 20 PCR technology. Template for PCR reactions were plasmid preparations containing the target genes. Overlapping oligos were designed to combine the C-terminal portion of one gene with the N terminal portion of the other gene. This process allows fusing the two genes together 25 in the correct reading frame after the appropriate PCR reactions have been performed. Initially one "fusion" oligo for each gene was put into a PCR reaction with a universal primer for the vector carrying the target gene. The complimentary "fusion" oligo was used with a 30 universal primer to PCR the other gene. At the end of this first PCR reaction, two separate products were obtained, with each individual gene having the fusion site present, creating enough overlap to drive the second round of PCR and create the desired fusion. In 35 the second round of PCR, the first two PCR products were combined along with universal primers and via the

overlapping regions, the full length fusion DNA sequence was produced.

The final PCR gene products were digested with restriction endonucleases XbaI and BamHI, and then 5 ligated into the vector pAMG21 having been also digested with the two restriction endonucleases.

Ligated DNA was transformed into competent host cells of *E. coli* strain 393. Clones were screened for the ability to produce the recombinant protein product and 10 to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate, sonic pellet, and supernatant were analyzed for expression of the fusion by Coomassie stained PAGE gels 15 and Western analysis with murine anti-OPG antibody.

Fc/huOPG [22-401]

Expression of the Fc/hu OPG [22-401] fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The cells have very large 20 inclusion bodies, and the majority of the product is in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

1318-48:

CAG CCC GGG TAA AAT GGA AAC GTT TCC TCC AAA ATA TCT TCA TT  
25 (SEQ ID NO:107)

1318-49:

CGT TTC CAT TTT ACC CGG GCT GAG CGA GAG GCT CTT CTG CGT GT  
(SEQ ID NO:108)

Fc/muOPG [22-401]

30 Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The cells have very large inclusion bodies, and the majority of the product is in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc 35 fusion:

1318-50:

CGC TCA GCC CGG GTA AAA TGG AAA CGT TGC CTC CAA AAT ACC TGC  
(SEQ ID NO:109)

1318-51:

CCA TTT TAC CCG GGC TGA GCG AGA GGC TCT TCT GCG TGT  
5 (SEQ ID NO:110)

muOPG [22-401]/Fc |

Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The amount of recombinant product was less than the OPG fusion 10 proteins having the Fc region in the N terminal position. Obvious inclusion bodies were not detected. Most of the product appeared to be in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

15 1318-54:

GAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC (SEQ ID NO:111)

1318-55:

CAG CTG CAG CTA AGC AGC TTA TTT TCA CGG ATT G (SEQ ID NO:112)

huOPG [22-401]/Fc

20 Expression of the fusion peptide was not detected on a Coomassie stained gel, although a faint Western positive signal was present. Obvious inclusion bodies were not detected. The following primers were used to prepare this OPG-Fc fusion:

25 1318-52:

AAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC (SEQ ID NO:113)

1318-53:

CAG CTG CAG CTA AGC AGC TTA TTT TCA CTG ATT GG (SEQ ID NO:114)

V. Human OPG met[22-401]-Fc fusion (P25A)

30 This construct combines a proline to alanine amino acid change at position 25 (P25A) with the huOPG met[22-401]-Fc fusion. The plasmid was digested with restriction endonucleases Clal and KpnI, which removes the N-terminal 28 codons of the gene, and the resulting 35 small (less than 200 base pair) fragment was gel purified. This fragment containing the proline to

alanine change was then ligated into plasmid pAMG21-huOPG [22-401]-Fc fusion which had been digested with the two restriction endonucleases. The ligated DNA was transformed into competent host cells of E. coli strain 5 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were 10 analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. The expression level of the fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The protein was in the insoluble 15 (pellet) fraction. The cells had large inclusion bodies.

W. Human OPG met[22-401] (P25A)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the 20 proline at position 25 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-84 and 1289-85 were annealed to form an oligo linker duplex with XbaI and KpnI cohesive 25 ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker also included an SpeI restriction site which was not present in the original sequence. The linker duplex was directionally inserted between the XbaI and KpnI sites in pAMG21-huOPG-22-401 using standard methods. 30 The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing 35 was performed to verify the DNA sequence of the HuOPG-

Met[22-401] (P25A) gene. The following oligonucleotides were used to generate the XbaI - KpnI linker:

Oligo #1289-84:

5'-CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC AAA ATA TCT TCA  
5 TTA TGA TGA AGA AAC TAG TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC  
GGG TAC -3' (SEQ ID NO:115)

Oligo #1289-85:

5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAC TAG TTT CTT CAT CAT  
AAT GAA GAT ATT TTG GAG CAA AAG TTT CCA TAT GTT ATT CCT CCT T-3'  
10 (SEQ ID NO:116)

X. Human OPG met[22-401] (P26A) and (P26D)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 26 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-86 and 1289-87 were annealed to form an oligo linker duplex with XbaI and SpeI cohesive ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker duplex was directionally inserted between the XbaI and SpeI sites in pAMG21-huOPG[22-401] (P25A) using standard methods. The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] (P26A) gene. One of the clones sequenced was found to have the proline at position 26 substituted by aspartic acid rather than alanine, and this clone was designated huOPG-met[22-401] (P26D). The following oligonucleotides were used to generate the XbaI - SpeI linker:

Oligo #1289-86:

35 5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC TAA ATA TCT  
TCA TTA TGA TGA AGA AA - 3' (SEQ ID NO:117)

## Oligo #1289-87:

5' - CTA GTT TCT TCA TCA TAA TGA AGA TAT TTA GCA GGA AAA GTT TCC  
ATA TGT TAT TCC TCC TT - 3' (SEQ ID NO:118)

Y. Human OPG met[22-194] (P25A)

- 5 A DNA sequence coding for an N-terminal methionine and amino acids 22 through 194 of human OPG with the proline at position 25 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows:
- 10 The plasmids pAMG21-huOPG[27-194] and pAMG21-huOPG[22-401] (P25A) were each digested with KpnI and BamHI endonucleases. The 450 bp fragment was isolated from pAMG21-huOPG[27-194] and the 6.1 kbp fragment was isolated from pAMG21-huOPG[22-401] (P25A). These
- 15 fragments were ligated together and introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-Met[22-194] (P25A) gene.
- 20

EXAMPLE 9Association of OPG Monomers

- CHO cells engineered to overexpress muOPG [22-401] were used to generate conditioned media for the analysis of secreted recombinant OPG using rabbit polyclonal anti-OPG antibodies. An aliquot of conditioned media was concentrated 20-fold, then analysed by reducing and non-reducing SDS-PAGE (Figure 15). Under reducing conditions, the protein migrated as a Mr 50-55 kd polypeptide, as would be predicted if the mature product was glycosylated at one or more of its consensus N-linked glycosylation sites. Surprisingly, when the same samples were analysed by non-reducing SDS-PAGE, the majority of the protein migrated as an approximately 100 kd polypeptide, twice the size of the reduced protein. In addition, there was
- 25
- 30
- 35

a smaller amount of the Mr 50-55 kd polypeptide. This pattern of migration on SDS-PAGE was consistent with the notion that the OPG product was forming dimers through oxidation of a free sulfhydryl group(s).

5       The predicted mature OPG polypeptide contains 23 cysteine residues, 18 of which are predicted to be involved in forming intrachain disulfide bridges which comprise the four cysteine-rich domains (Figure 12A). The five remaining C-terminal cysteine residues are not  
10 involved in secondary structure which can be predicted based upon homology with other TNFR family members. Overall there is a net uneven number of cysteine residues, and it is formally possible that at least one residue is free to form an intermolecular disulfide  
15 bond between two OPG monomers.

To help elucidate patterns of OPG kinesis and monomer association, a pulse-chase labelling study was performed. CHO cells expressing muOPG [22-401] were metabolically labelled as described above in serum-free  
20 medium containing  $^{35}\text{S}$  methionine and cysteine for 30 min. After this period, the media was removed, and replaced with complete medium containing unlabelled methionine and cysteine at levels approximately 2,000-fold excess to the original concentration of  
25 radioactive amino acids. At 30 min, 1hr, 2 hr, 4 hr, 6 hr and 12 hr post addition, cultures were harvested by the removal of the conditioned media, and lysates of the conditioned media and adherent monolayers were prepared. The culture media and cell lysates were  
30 clarified as described above, and then immunoprecipitated using anti-OPG antibodies as described above. After the immunoprecipitates were washed, they were released by boiling in non-reducing SDS-PAGE buffer then split into two equal halves. To  
35 one half, the reducing agent  $\beta$ -mercaptoethanol was added

to 5% (v/v) final concentration, while the other half was maintained in non-reducing conditions. Both sets of immunoprecipitates were analysed by SDS-PAGE as described above, then processed for autoradiography and exposed to film. The results are shown in Figure 16.

The samples analysed by reducing SDS-PAGE are depicted in the bottom two panels. After synthesis, the OPG polypeptide is rapidly processed to a slightly larger polypeptide, which probably represents modification by N-linked glycosylation. After approximately 1-2 hours, the level of OPG in the cell decreases dramatically, and concomitantly appears in the culture supernatant. This appears to be the result of the vectoral transport of OPG from the cell into the media over time, consistent with the notion that OPG is a naturally secreted protein. Analysis of the same immunoprecipitates under nonreducing conditions reveals the relationship between the formation of OPG dimers and secretion into the conditioned media (Figure 16, upper panels). In the first 30-60 minutes, OPG monomers are processed in the cell by apparent glycosylation, followed by dimer formation. Over time, the bulk of OPG monomers are driven into dimers, which subsequently disappear from the cell. Beginning about 60 minutes after synthesis, OPG dimers appear in the conditioned media, and accumulate over the duration of the experiment. Following this period, OPG dimers are formed, which are then secreted into the culture media. OPG monomers persist at a low level inside the cell over time, and small amounts also appear in the media. This does not appear to be the result of breakdown of covalent OPG dimers, but rather the production of substoichiometric amounts of monomers in the cell and subsequent secretion.

Recombinantly produced OPG from transfected CHO cells appears to be predominantly a dimer. To determine

if dimerization is a natural process in OPG synthesis, we analysed the conditioned media of a cell line found to naturally express OPG. The CTLL-2 cell line, a murine cytotoxic T lymphocytic cell line (ATCC accession no. TIB-214), was found to express OPG mRNA in a screen of tissue and cell line RNA. The OPG transcript was found to be the same as the cloned and sequenced 2.5-3.0 kb RNA identified from kidney and found to encode a secreted molecule. Western blot analysis of conditioned media obtained from CTLL-2 cells shows that most, if not all, of the OPG secreted is a dimer (Figure 17). This suggests that OPG dimerization and secretion is not an artifact of overexpression in a cell line, but is likely to be the main form of the product as it is produced by expressing cells.

Normal and transgenic mouse tissues and serum were analysed to determine the nature of the OPG molecule expressed in OPG transgenic mice. Since the rat OPG cDNA was expressed under the control of a hepatocyte control element, extracts made from the parenchyma of control and transgenic mice under non-reducing conditions were analysed (Figure 18). In extract from transgenic, but not control mice, OPG dimers are readily detected, along with substoichiometric amounts of monomers. The OPG dimers and monomers appear identical to the recombinant murine protein expressed in the genetically engineered CHO cells. This strongly suggests that OPG dimers are indeed a natural form of the gene product, and are likely to be key active components. Serum samples obtained from control and transgenic mice were similarly analysed by western blot analysis. In control mice, the majority of OPG migrates as a dimer, while small amounts of monomer are also detected. In addition, significant amounts of a larger OPG related protein is detected, which migrates with a

relative molecular mass consistent with the predicted size of a covalently-linked trimer. Thus, recombinant OPG is expressed predominantly as a dimeric protein in OPG transgenic mice, and the dimer form may be the 5 basis for the osteopetrosis phenotype in OPG mice. OPG recombinant protein may also exist in higher molecular weight "trimeric" forms.

To determine if the five C-terminal cysteine residues of OPG play a role in homodimerization, the 10 murine OPG codons for cysteine residues 195 (C195), C202, C277, C319, and C400 were changed to serine using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as described above. The muOPG gene was subcloned between the Not I and Xba I 15 sites of the pcDNA 3.1 (+) vector (Invitrogen, San Diego, CA). The resulting plasmid, pcDNA3.1-muOPG, and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the 20 reaction is then transfected into competent *E. coli* XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to change the codon for cysteine residue 195 to serine of the murine 25 OPG gene, resulting in the production of a muOPG [22-401] C195S protein:

. 1389-19:

5' -CAC GCA AAA GTC GGG AAT AGA TGT CAC-3' (SEQ ID NO:150)

1406-38:

30 5' -GTG ACA TCT ATT CCC GAC TTT TGC GTG-3' (SEQ ID NO:151)

The following primer pairs were used to change the codon for cysteine residue 202 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C202S protein:

35 1389-21:

5' -CAC CCT GTC GGA AGA GGC CTT CTT C-3' (SEQ ID NO:152)

1389-22:

5' -GAA GAA GGC CTC TTC CGA CAG GGT G-3' (SEQ ID NO:153)

The following primer pairs were used to change the codon for cysteine residue 277 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C277S protein:

1389-23:

5' -TGA CCT CTC GGA AAG CAG CGT GCA-3' (SEQ ID NO:154)

1389-24:

10 5' -TGC ACG CTG CTT TCC GAG AGG TCA-3' (SEQ ID NO:155)

The following primer pairs were used to change the codon for cysteine residue 319 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C319S protein:

15 1389-17:

5' -CCT CGA AAT CGA GCG AGC AGC TCC-3' (SEQ ID NO:156)

1389-18:

5' -CGA TTT CGA GGT CTT TCT CGT TCT C-3' (SEQ ID NO:157)

20 The following primer pairs were used to change the codon for cysteine residue 400 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C400S protein:

1406-72:

5' -CCG TGA AAA TAA GCT CGT TAT AAC TAG GAA TGG-3'

25 (SEQ ID NO:158)

1406-75:

5' -CCA TTC CTA GTT ATA ACG AGC TTA TTT TCA CGG-3'

(SEQ ID NO:159)

30 Each resulting muOPG [22-401] plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed in the in vitro osteoclast forming assay described in example 11.

35 Conditioned media from each transfectant was analysed

by non-reducing SDS-PAGE and western blotting with anti-OPG antibodies.

Mutation of any of the five C-terminal cysteine residues results in the production of predominantly (>90%) monomeric 55 kd OPG molecules. This strongly suggests that the C-terminal cysteine residues together play a role in OPG homodimerization.

C-terminal OPG deletion mutants were constructed to map the region(s) of the OPG C-terminal domain which are important for OPG homodimerization. These OPG mutants were constructed by PCR amplification using primers which introduce premature stop translation signals in the C-terminal region of murine OPG. The 5' oligo was designed to the MuOPG start codon (containing a HindIII restriction site) and the 3' oligonucleotides (containing a stop codon and XhoI site) were designed to truncate the C-terminal region of muOPG ending at either threonine residue 200 (CT 200), proline 212 (CT212), glutamic acid 293 (CT-293), or serine 355 (CT-355).

The following primers were used to construct muOPG [22-200]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
25 (SEQ ID NO:160)

1391-91:

5' -CCT CTC TCG AGT CAG GTG ACA TCT ATT CCA CAC TTT TGC GTG GC-3'  
(SEQ ID NO:161)

The following primers were used to construct muOPG  
30 [22-212]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
(SEQ ID NO:162)

1391-90:

35 5' -CCT CTC TCG AGT CAA GGA ACA GCA AAC CTG AAG AAG GC -3'  
(SEQ ID NO:163)

The following primers were used to construct muOPG [22-293]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
5 (SEQ ID NO:164)

1391-89:

5' - CCT CTC TCG AGT CAC TCT GTG GTG AGG TTC GAG TGG CC-3'  
(SEQ ID NO:165)

The following primers were used to construct muOPG  
10 [22-355]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3'  
(SEQ ID NO:166)

1391-88:

15 5' CCT CTC TCG AGT CAG GAT GTT TTC AAG TGC TTG AGG GC-3'  
(SEQ ID NO:167)

Each resulting muOPG-CT plasmid containing the appropriate truncation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified  
20 from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11. The conditioned medias were also analysed by non-reducing SDS-PAGE and western blotting using anti-OPG  
25 antibodies.

Truncation of the C-terminal region of OPG effects the ability of OPG to form homodimers. CT 355 is predominantly monomeric, although some dimer is formed. CT 293 forms what appears to be equal molar amounts of monomer and dimer, and also high molecular weight aggregates. However, CT 212 and CT 200 are monomeric.

#### EXAMPLE 10

##### Purification of OPG

###### A. Purification of mammalian OPG-Fc Fusion Proteins

35 5 L of conditioned media from 293 cells expressing an OPG-Fc fusion protein were prepared as follows. A

frozen sample of cells was thawed into 10 ml of 293S media (DMEM-high glucose, 1x L-glutamine, 10% heat inactivated fetal bovine serum (FBS) and 100 ug/ml hygromycin) and fed with fresh media after one day.

5 After three days, cells were split into two T175 flasks at 1:10 and 1:20 dilutions. Two additional 1:10 splits were done to scale up to 200 T175 flasks. Cells were at 5 days post-thawing at this point. Cells were grown to near confluence (about three days) at which time serum-containing media was aspirated, cells were washed one time with 25 ml PBS per flask and 25 ml of SF media (DMEM-high glucose, 1x L-glutamine) was added to each flask. Cells were maintained at 5% CO<sub>2</sub> for three days at which point the media was harvested, centrifuged,

10 and filtered through 0.45m cellulose nitrate filters (Corning).

15

OPG-Fc fusion proteins were purified using a Protein G Sepharose column (Pharmacia) equilibrated in PBS. The column size varied depending on volume of starting media. Conditioned media prepared as described above was loaded onto the column, the column washed with PBS, and pure protein eluted using 100mM glycine pH 2.7. Fractions were collected into tubes containing 1M Tris pH 9.2 in order to neutralize as quickly as possible. Protein containing fractions were pooled, concentrated in either an Amicon Centricon 10 or Centriprep 10 and diafiltered into PBS. The pure protein is stored at -80°C.

Murine [22-401]-Fc, Murine [22-180]-Fc, Murine [22-194]-Fc, human [22-401]-Fc and human [22-201]Fc were purified by this procedure. Murine [22-185]-Fc is purified by this procedure.

B. Preparation of anti-OPG antibodies

Three New Zealand White rabbits (5-8 lbs initial wt) were injected subcutaneously with muOPG[22-401]-Fc fusion protein. Each rabbit was immunized on day 1 with

50 µg of antigen emulsified in an equal volume of Freunds complete adjuvant. Further boosts (Days 14 and 28) were performed by the same procedure with the substitution of Freunds incomplete adjuvant. Antibody  
5 titers were monitored by EIA. After the second boost, the antisera revealed high antibody titers and 25ml production bleeds were obtained from each animal. The sera was first passed over an affinity column to which murine OPG-Fc had be immobilized. The anti-OPG  
10 antibodies were eluted with Pierce Gentle Elution Buffer containing 1% glacial acetic acid. The eluted protein was then dialyzed into PBS and passed over a Fc column to remove any antibodies specific for the Fc portion of the OPG fusion protein. The run through  
15 fractions containing anti-OPG specific antibodies were dialyzed into PBS.

C. Purification of murine OPG[22-401]

Antibody Affinity Chromatography

Affinity purified anti-OPG antibodies were  
20 diafiltered into coupling buffer (0.1M sodium carbonate pH 8.3, 0.5M NaCl), and mixed with CNBr-activated sepharose beads (Pharmacia) for two hours at room temperature. The resin was then washed with coupling buffer extensively before blocking unoccupied sited  
25 with 1M ethanolamine (pH 8.0) for two hours at room temperature. The resin was then washed with low pH (0.1M sodium acetate pH 4.0, 0.5M NaCl) followed by a high pH wash (0.1M Tris-HCl pH 8.0, 0.5M NaCl). The last washes were repeated three times. The resin was  
30 finally equilibrated with PBS before packing into a column. Once packed, the resin was washed with PBS. A blank elution was performed with 0.1M glycine-HCl, pH 2.5), followed by re-equilibration with PBS.

Concentrated conditioned media from CHO cells  
35 expressing muOPG[22-410] was applied to the column at a

low flow rate. The column was washed with PBS until UV absorbance measured at 280nm returned to baseline. The protein was eluted from the column first with 0.1M glycine-HCl (pH 2.5), re-equilibrated with PBS, and 5 eluted with a second buffer (0.1M CAPS, pH 10.5), 1M NaCl). The two elution pools were diafiltered separately into PBS and sterile filtered before freezing at -20°C.

Conventional Chromatography

10 CHO cell conditioned media was concentrated 23x in an Amicon spiral wound cartridge (S10Y10) and diafiltered into 20mM tris pH 8.0. The diafiltered media was then applied to a Q-sepharose HP (Pharmacia) column which had been equilibrated with 20mM tris pH 8.0. The column was then washed until absorbence at 280 nm reached baseline. Protein was eluted with a 20 column volume gradient of 0-300 mM NaCl in tris pH 8.0. OPG was detected using a western blot of column fractions.

20 Fractions containing OPG were pooled and brought to a final concentration of 300 mM NaCl, 0.2 mM DTT. A NiNTA superose (Qiagen) column was equilibrated with 20mM tris pH 8.0, 300 mM NaCl, 0.2 mM DTT after which the pooled fractions were applied. The column was 25 washed with equilibration buffer until baseline absorbence was reached. Proteins were eluted from the column with a 0-30mM Imidazole gradient in equilibration buffer. Remaining proteins were washed off the column with 1M Imidazole. Again a western blot 30 was used to detect OPG containing fractions.

Pooled fractions from the NiNTA column were dialyzed into 10 mM potassium phosphate pH 7.0, 0.2mM DTT. The dialyzed pool was then applied to a ceramic hydroxyapatite column (Bio-Rad) which had been 35 equilibrated in 10mM phosphate buffer. After column washing, the protein was eluted with a 10-100 mM

potassium phosphate gradient over 20 column volumes. This was then followed by a 20 column volume gradient of 100-400 mM phosphate.

OPG was detected by coomassie blue staining of  
5 SDS-polyacrylamide gels and by western blotting. Fractions were pooled and diafiltered onto PBS and frozen at -80°C. The purified protein runs as a monomer and will remain so after diafiltration into PBS. The monomer is stable when stored frozen or at pH 5 at 4°C.  
10 However if stored at 4°C in PBS, dimers and what appears to be trimers and tetramers will form after one week.

D. Purification of human OPG met[22-401] from E. coli

The bacterial cell paste was suspended into 10 mM  
15 EDTA to a concentration of 15% (w/v) using a low shear homogenizer at 5°C. The cells were then disrupted by two homogenizations at 15,000 psi each at 5°C. The resulting homogenate was centrifuged at 5,000 x g for one hour at 5°C. The centrifugal pellet was washed by  
20 low shear homogenization into water at the original homogenization volume followed by centrifugation as before. The washed pellet was then solubilized to 15% (w/v) by a solution of (final concentration) 6 M guanidine HCl, 10 mM dithiothreitol, 10 mM TrisHCl, pH  
25 8.5 at ambient temperature for 30 minutes. This solution was diluted 30-fold into 2M urea containing 50 mM CAPS, pH 10.5, 1 mM reduced glutathione and then stirred for 72 hours at 5°C. The OPG was purified from this solution at 25°C by first adjustment to pH 4.5  
30 with acetic acid and then chromatography over a column of SP-HP Sepharose resin equilibrated with 25 mM sodium acetate, pH 4.5. The column elution was carried out with a linear sodium chloride gradient from 50 mM to 550 mM in the same buffer using 20 column volumes at a  
35 flow rate of 0.1 column volumes/minute. The peak fractions containing only the desired OPG form were

pooled and stored at 5°C or buffer exchanged into phosphate buffered saline, concentrated by ultrafiltration, and then stored at 5°C. This material was analyzed by reverse phase HPLC, SDS-PAGE, limulus amebocyte lysate assay for the presence of endotoxin, and N-terminal sequencing. In addition, techniques such as mass spectrometry, pH/temperature stability, fluorescence, circular dichroism, differential scanning calorimetry, and protease profiling assays may also be used to examine the folded nature of the protein.

**EXAMPLE 11**

**Biological Activity of Recombinant OPG**

Based on histology and histomorphometry, it appeared that hepatic overexpression of OPG in transgenic mice markedly decreased the numbers of osteoclasts leading to a marked increase in bone tissue (see Example 4). To gain further insight into potential mechanism(s) underlying this *in vivo* effect, various forms of recombinant OPG have been tested in an *in vitro* culture model of osteoclast formation (osteoclast forming assay). This culture system was originally devised by Udagawa (Udagawa *et al.* Endocrinology 125, 1805-1813 (1989), Proc. Natl. Acad. Sci. USA 87, 7260-7264 (1990)) and employs a combination of bone marrow cells and cells from bone marrow stromal cell lines. A description of the modification of this culture system used for these studies has been previously published (Lacey *et al.* Endocrinology 136, 2367-2376 (1995)). In this method, bone marrow cells, flushed from the femurs and tibiae of mice, are cultured overnight in culture media (alpha MEM with 10% heat inactivated fetal bovine serum) supplemented with 500 U/ml CSF-1 (colony stimulating factor 1, also called M-CSF), a hematopoietic growth factor specific for cells of the monocyte/macrophage family lineage. Following this incubation, the non-adherent cells are collected,

subjected to gradient purification, and then cocultured with cells from the bone marrow cell line ST2 ( $1 \times 10^6$  non-adherent cells :  $1 \times 10^5$  ST2 cells/ ml media). The media is supplemented with dexamethasone (100 nM) and 5 the biologically-active metabolite of vitamin D3 known as 1,25 dihydroxyvitamin D3 (1,25 (OH)<sub>2</sub> D<sub>3</sub>, 10 nM). To enhance osteoclast appearance, prostaglandin E2 (250 nM) is added to some cultures. The coculture period usually ranges from 8 - 10 days and the media, with all 10 of the supplements freshly added, is renewed every 3-4 days. At various intervals, the cultures are assessed for the presence of tartrate acid phosphatase (TRAP) using either a histochemical stain (Sigma Kit # 387A, Sigma, St. Louis, MO) or TRAP solution assay. The TRAP 15 histochemical method allows for the identification of osteoclasts phenotypically which are multinucleated (> 3 nuclei) cells that are also TRAP+. The solution assay involves lysing the osteoclast-containing cultures in a citrate buffer (100 mM, pH 5.0) containing 0.1% Triton 20 X-100. Tartrate resistant acid phosphatase activity is then measured based on the conversion of p-nitrophenylphosphate (20 nM) to p-nitrophenol in the presence of 80 mM sodium tartrate which occurs during a 3-5 minute incubation at RT. The reaction is terminated 25 by the addition of NaOH to a final concentration of 0.5 M. The optical density at 405 nm is measured and the results are plotted.

Previous studies (Udagawa *et al. ibid*) using the osteoclast forming assay have demonstrated that these 30 cells express receptors for <sup>125</sup>I-calcitonin (autoradiography) and can make pits on bone surfaces, which when combined with TRAP positivity confirm that the multinucleated cells have an osteoclast phenotype. Additional evidence in support of the osteoclast 35 phenotype of the multinucleated cells that arise in

vitro in the osteoclast forming assay are that the cells express  $\alpha v$  and  $\beta 3$  integrins by immunocytochemistry and calcitonin receptor and TRAP mRNA by *in situ* hybridization (ISH).

5       The huOPG [22-401]-Fc fusion was purified from CHO cell conditioned media and subsequently utilized in the osteoclast forming assay. At 100 ng/ml of huOPG [22-401]-Fc, osteoclast formation was virtually 100% inhibited (Figure 19A). The levels of TRAP measured in  
10 lysed cultures in microtitre plate wells were also inhibited in the presence of OPG with an ID<sub>50</sub> of approximately 3 ng/ml (Figure 20). The level of TRAP activity in lysates appeared to correlate with the relative number of osteoclasts seen by TRAP  
15 cytochemistry (compare Figures 19A-19G and 20). Purified human IgG1 and TNF- $\alpha$  inhibitor were also tested in this model and were found to have no inhibitory or stimulatory effects suggesting that the inhibitory effects of the huOPG [22-401]-Fc were due to  
20 the OPG portion of the fusion protein. Additional forms of the human and murine molecules have been tested and the cumulative data are summarized in Table 3.

Table 3  
Effects of various OPG forms on in vitro  
osteoclast formation

	<u>OPG Construct</u>	<u>Relative Bioactivity in vitro</u>
	muOPG [22-401]-Fc	+++
	muOPG [22-194]-Fc	+++
	muOPG [22-185]-Fc	++
10	muOPG [22-180]-Fc	-
	muOPG [22-401]	+++
	muOPG [22-401] C195	+++
	muOPG [22-401] C202	+
	muOPG [22-401] C277	-
15	muOPG [22-401] C319	+
	muOPG [22-401] C400	+
	muOPG [22-185]	-
	muOPG [22-194]	++
	muOPG [22-200]	++
20	muOPG [22-212]	-
	muOPG [22-293]	+++
	muOPG [22-355]	+++
	huOPG [22-401]-Fc	+++
25	huOPG [22-201]-Fc	+++
	huOPG [22-401]-Fc P26A	+++
	huOPG [22-401]-Fc Y28F	+++
	huOPG [22-401]	+++
	huOPG [27-401]-Fc	++
30	huOPG [29-401]-Fc	++
	huOPG [32-401]-Fc	+/-
	+++ , ED <sub>50</sub> = 0.4-2 ng/ml	
	++ , ED <sub>50</sub> = 2-10 ng/ml	
35	+ , ED <sub>50</sub> = 10-100 ng/ml	
	- , ED <sub>50</sub> > 100 ng/ml	

The cumulative data suggest that murine and human OPG amino acid sequences 22-401 are fully active in vitro, when either fused to the Fc domain, or unfused. They inhibit in a dose-dependent manner and possess 5 half-maximal activities in the 2-10 ng/ml range. Truncation of the murine C-terminus at threonine residue 180 inactivates the molecule, whereas truncations at cysteine 185 and beyond have full activity. The cysteine residue located at position 185 10 is predicted to form an SS3 bond in the domain 4 region of OPG. Removal of this residue in other TNFR-related proteins has previously been shown to abrogate biological activity (Yan *et al.* (1994), *J. Biol. Chem.* 266: 12099-104). Our finding that muOPG[22-180]-Fc is 15 inactive while muOPG[22-185]-Fc is active is consistent with these findings. This suggests that amino acid residues 22-185 define a region for OPG activity.

These findings indicate that like transgenically-expressed OPG, recombinant OPG also 20 suppressed osteoclast formation as tested in the osteoclast forming assay. Time course experiments examining the appearance of TRAP+ cells,  $\beta$ 3+ cells, F480+ cells in cultures continuously exposed to OPG demonstrate that OPG blocks the appearance TRAP+ and 25  $\beta$ 3+ cells, but not F480+ cells. In contrast, TRAP+ and  $\beta$ 3+ cells begin to appear as early as day 4 following culture establishment in control cultures. Only F480+ cells can be found in OPG-treated cultures and they 30 appear to be present at qualitatively the same numbers as the control cultures. Thus, the mechanism of OPG effects in vitro appears to involve a blockade in osteoclast differentiation at a step beyond the appearance of monocyte-macrophages but before the 35 appearance of cells expressing either TRAP or  $\beta$ 3 integrins. Collectively these findings indicate that

OPG does not interfere with the general growth and differentiation of monocyte-macrophage precursors from bone marrow, but rather suggests that OPG specifically blocks the selective differentiation of osteoclasts  
5 from monocyte-macrophage precursors.

To determine more specifically when in the osteoclast differentiation pathway that OPG was inhibitory, a variation of the in vitro culture method was employed. This variation, described in (Lacey *et al. supra*), employs bone marrow macrophages as  
10 osteoclast precursors. The osteoclast precursors are derived by taking the nonadherent bone marrow cells after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000  
15 - 2,000 U/ml CSF-1. Following 4 days of culture, termed the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/ml CSF-1.  
20 This 2 day period is called the intermediate differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells ( $1 \times 10^5$  cell/ml), dexamethasone (100 nM) and 1,25 (OH)<sub>2</sub> D<sub>3</sub> (10 nM) are added for the last 8 days for what is termed the  
25 terminal differentiation period. Test agents can be added during this terminal period as well. Acquisition of phenotypic markers of osteoclast differentiation are acquired during this terminal period (Lacey *et al. ibid*).

30 huOPG [22-401]-Fc (100 ng/ml) was tested for its effects on osteoclast formation in this model by adding it during either the intermediate, terminal or, alternatively, both differentiation periods. Both TRAP cytochemistry and solution assays were performed. The  
35 results of the solution assay are shown in Figure 21.

HuOPG [22-401]-Fc inhibited the appearance of TRAP activity when added to both the intermediate and terminal or only the terminal differentiation phases. When added to the intermediate phase and then removed from the cultures by rinsing, huOPG [22-401]-Fc did not block the appearance of TRAP activity in culture lysates. The cytochemistry results parallel the solution assay data. Collectively, these observations indicate that huOPG [22-401]-Fc only needs to be present during the terminal differentiation period for it to exert its all of its suppressive effects on osteoclast formation.

B. In vivo IL-1- $\alpha$  and IL-1- $\beta$  challenge experiments

IL-1 increases bone resorption both systemically and locally when injected subcutaneously over the calvaria of mice (Boyce *et al.* (1989), *Endocrinology* 125: 1142-50). The systemic effects can be assessed by the degree of hypercalcemia and the local effects histologically by assessing the relative magnitude of the osteoclast-mediated response. The aim of these experiments was to determine if recombinant muOPG [22-401]-Fc could modify the local and/or systemic actions of IL-1 when injected subcutaneously over the same region of the calvaria as IL-1.

IL-1  $\beta$  experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group: IL-1 treated animals (mice received 1 injection/day of 2.5 ug of IL-1- $\beta$ ); Low dose muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 1  $\mu$ g of muOPG [22-401]-Fc); Low dose muopg [22-401]-Fc and IL-1- $\beta$ ; High dose muOPG [22-401]-Fc treated animals (mice receive 3 injections/day of 10  $\mu$ g muOPG [22-401]-Fc); High dose muOPG [22-401]-Fc and

IL-1- $\beta$ . All mice received the same total number of injections of either active factor or vehicle (0.1% bovine serum albumin in phosphate buffered saline). All groups are sacrificed on the day after the last  
5 injection. The weights and blood ionized calcium levels are measured before the first injections, four hours after the second injection and 24 hours after the third IL-1 injection, just before the animals were sacrificed. After sacrifice the calvaria were removed  
10 and processed for paraffin sectioning.

IL-1  $\alpha$  experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group; IL-1- $\alpha$  treated animals (mice  
15 received 1 injection/day of 5  $\mu$ g of IL-1- $\alpha$ ); Low dose muOPG [22-401]-Fc treated animals (mice received 1 injection/day of 10  $\mu$ g of muOPG [22-401]-Fc; Low dose muopg [22-401]-Fc and IL-1- $\alpha$ , (dosing as above); High dose muopg [22-401]-Fc treated animals (mice received 3  
20 injections/day of 10  $\mu$ g muOPG [22-401]-Fc; High dose muOPG [22-401]-Fc and IL-1- $\alpha$ . All mice received the same number of injections/day of either active factor or vehicle. All groups were sacrificed on the day after the last injection. The blood ionized calcium levels  
25 were measured before the first injection, four hours after the second injection and 24 hours after the third IL-1 injection, just before the animals were sacrificed. The animal weights were measured before the first injection, four hours after the second injection and 24 hours after the third IL-1 injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin  
30 sectioning.

Histological methods

Calvarial bone samples were fixed in zinc formalin, decalcified in formic acid, dehydrated through ethanol and mounted in paraffin. Sections (5 $\mu$ m thick) were cut through the calvaria adjacent to the lambdoid suture and stained with either hematoxylin and eosin or reacted for tartrate resistant acid phosphatase activity (Sigma Kit# 387A) and counterstained with hematoxylin. Bone resorption was assessed in the IL-1  $\alpha$  treated mice by histomorphometric methods using the Osteomeasure (Osteometrics, Atlanta, GA) by tracing histologic features onto a digitizer platen using a microscope-mounted camera lucida attachment. Osteoclast numbers, osteoclast lined surfaces, and eroded surfaces were determined in the marrow spaces of the calvarial bone. The injected and non-injected sides of the calvaria were measured separately.

Results

IL-1  $\alpha$  and IL-1  $\beta$  produced hypercalcemia at the doses used, particularly on the second day, presumably by the induction of increased bone resorption systemically. The hypercalcemic response was blocked by muOPG [22-401]-Fc in the IL-1 beta treated mice and significantly diminished in mice treated with IL-1- $\alpha$ , an effect most apparent on day 2 (Figure 22A-22B).

Histologic analysis of the calvariae of mice treated with IL-1- $\alpha$  and beta shows that IL-1 treatments alone produce a marked increase in the indices of bone resorption including: osteoclast number, osteoclast lined surface, and eroded surface (surfaces showing deep scalloping due to osteoclastic action (Figure 23B). In response to IL-1  $\alpha$  or IL-1  $\beta$ , the increases in bone resorption were similar on the injected and non-

injected sides of the calvaria. Muopg [22-401]-Fc injections reduced bone resorption in both IL-1- $\alpha$  and beta treated mice and in mice receiving vehicle alone but this reduction was seen only on the muopg [22-401]-Fc injected sides of the calvariae.

The most likely explanation for these observations is that muOPG [22-401]-Fc inhibited bone resorption, a conclusion supported by the reduction of both the total osteoclast number and the percentage of available bone surface undergoing bone resorption, in the region of the calvaria adjacent to the muOPG [22-401]-Fc injection sites. The actions of muOPG [22-401]-Fc appeared to be most marked locally by histology, but the fact that muOPG [22-401]-Fc also blunted IL-1 induced hypercalcemia suggests that muOPG [22-401]-Fc has more subtle effects on bone resorption systemically.

C. Systemic Effects of muOPG [22-401]-Fc in Growing Mice

Male BDF1 mice aged 3-4 weeks, weight range 9.2-15.7g were divided into groups of ten mice per group. These mice were injected subcutaneously with saline or muOPG [22-401]-Fc 2.5mg/kg bid for 14 days (5mg/kg/day). The mice were radiographed before treatment, at day 7 and on day 14. The mice were sacrificed 24 hours after the final injection. The right femur was removed, fixed in zinc formalin, decalcified in formic acid and embedded in paraffin. Sections were cut through the mid region of the distal femoral metaphysis and the femoral shaft. Bone density, by histomorphometry, was determined in six adjacent regions extending from the metaphyseal limit of the growth plate, through the primary and secondary spongiosa and into the femoral diaphysis (shaft). Each region was 0.5 x 0.5 mm<sup>2</sup>.

Radiographic changes

After seven days of treatment there was evidence of a zone of increased bone density in the spongiosa associated with the growth plates in the OPG treated 5 mice relative to that seen in the controls. The effects were particularly striking in the distal femoral and the proximal tibial metaphyses (Figure 24A-24B). However bands of increased density were also apparent in the vertebral bodies, the iliac crest and the distal 10 tibia. At 14 days, the regions of opacity had extended further into the femoral and tibial shafts though the intensity of the radio-opacity was diminished. Additionally, there were no differences in the length 15 of the femurs at the completion of the experiment or in the change in length over the duration of the experiment implying that OPG does not alter bone growth.

Histological Changes

The distal femoral metaphysis showed increased 20 bone density in a regions 1.1 to 2.65 mm in distance from the growth plate (Figures 25 and 26A-26B). This is a region where bone is rapidly removed by osteoclast-mediated bone resorption in mice. In these rapidly growing young mice, the increase in bone in this region 25 observed with OPG treatment is consistent with an inhibition of bone resorption.

D. Effects of Osteoprotegerin on Bone Loss Induced by Ovariectomy in the Rat

Twelve week old female Fisher rats were 30 ovariectomized (OVX) or sham operated and dual xray absorptiometry (DEXA) measurements made of the bone density in the distal femoral metaphysis. After 3 days recovery period, the animals received daily injections for 14 days as follows: Ten sham operated animals 35 received vehicle (phosphate buffered saline); Ten OVX animals received vehicle (phosphate buffered saline);

Six OVX animals received OPG-Fc 5mg/kg SC; Six OVX animals received pamidronate (PAM) 5mg/kg SC; Six OVX animals received estrogen (ESTR) 40ug/kg SC. After 7 and 14 days treatment the animals had bone density measured by DEXA. Two days after the last injection the animals were killed and the right tibia and femur removed for histological evaluation.

The DEXA measurements of bone density showed a trend to reduction in the bone density following ovariectomy that was blocked by OPG-Fc. Its effects were similar to the known antiresorptive agents estrogen and pamidronate. (Figure 27). The histomorphometric analysis confirmed these observations with OPG-Fc treatment producing a bone density that was significantly higher in OVX rats than that seen in untreated OVX rats (Figure 28). These results confirm the activity of OPG in the bone loss associated with withdrawal of endogenous estrogen following ovariectomy.

20           In vivo Summary

The in vivo actions of recombinant OPG parallel the changes seen in OPG transgenic mice. The reduction in osteoclast number seen in the OPG transgenic is reproduced by injecting recombinant OPG locally over the calvaria in both normal mice and in mice treated with IL-1  $\alpha$  or IL-1  $\beta$ . The OPG transgenic mice develop an osteopetrotic phenotype with progressive filling of the marrow cavity with bone and unremodelled cartilage extending from the growth plates from day 1 onward after birth. In normal three week old (growing) mice, OPG treatments also led to retention of bone and unremodelled cartilage in regions of endochondral bone formation, an effect observed radiographically and confirmed histologically. Thus, recombinant OPG produces phenotypic changes in normal animals similar

to those seen in the transgenic animals and the changes are consistent with OPG-induced inhibition of bone resorption. Based on in vitro assays of osteoclast formation, a significant portion of this inhibition is 5 due to impaired osteoclast formation. Consistent with this hypothesis, OPG blocks ovariectomy-induced osteoporosis in rat. Bone loss in this model is known to be mediated by activated osteoclasts, suggesting a role for OPG in treatment of primary osteoporosis.

10

**EXAMPLE 12**

Pegylation Derivatives of OPG  
Preparation of N-terminal PEG-OPG conjugates by  
reductive alkylation

HuOPG met [22-194] P25A was buffer exchanged into 15 25-50 mM NaOAc, pH 4.5-4.8 and concentrated to 2-5 mg/ml. This solution was used to conduct OPG reductive alkylation with monofunctional PEG aldehydes at 5-7 C. PEG monofunctional aldehydes, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were 20 added to the OPG solution as solids in amounts constituting 2-4 moles of PEG aldehyde per mole of OPG. After dissolution of polymer into the protein solution, sodium cyanoborohydride was added to give a final concentration of 15 to 20 mM in the reaction mixture 25 from 1-1.6 M freshly prepared stock solution in cold DI water. The progress of the reaction and the extent of OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the 30 reaction was allowed to proceed for 16-18 hours, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 35 with a linear gradient to 0.75M NaCl over 25 column

volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE. By N-terminal sequencing, it was determined that the monoPEG-OPG conjugate, the major reaction product in most cases, was 98% N-terminally PEG-modified OPG.

This procedure was generally used to prepare the following N-terminal PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A: 5 kD monoPEG, 10 kD mono branched PEG, 12 kD monoPEG, 20 kD monoPEG, 20 kD mono branched PEG, 25 kD monoPEG, 31 kD monoPEG, 57 kD monoPEG, 12 kD diPEG, 25 kD diPEG, 31 kD diPEG, 57 kD diPEG, 25 kD triPEG).

Preparation of PEG-OPG conjugates by acylation

HuOPG met [22-194] P25A was buffer exchanged into 50 mM BICINE buffer, pH 8 and concentrated to 2-3 mg/ml. This solution was used to conduct OPG acylation with monofunctional PEG N-hydroxysuccinimidyl esters at room temperature. PEG N-hydroxysuccinimidyl esters, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 4-8 moles of PEG N-hydroxysuccinimidyl ester per mole of OPG. The progress of the reaction and the extent of OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 1 hour, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE.

This procedure was generally used to prepare the following PEG-OPG conjugates: 5 kD polyPEG, 20 kD polyPEG, 40 kD poly branched PEG, 50 kD poly PEG.

Preparation of dimeric PEG-OPG

5        HuOPG met [22-194] P25A is prepared for thiolation at 1-3 mg/ml in a phosphate buffer at near neutral pH. S-acetyl mecaptosuccinic anhydride (AMSA) is added in a 3-7 fold molar excess while maintaining pH at 7.0 and the rxn stirred at 4°C for 2 hrs. The monothiolated-OPG  
10      is separated from unmodified and polythiolated OPG by ion exchange chromatography and the protected thiol deprotected by treatment with hydroxylamine. After deprotection, the hydroxylamine is removed by gel filtration and the resultant monothiolated-OPG is  
15      subjected to a variety of thiol specific crosslinking chemistries. To generate a disulfide bonded dimer, the thiolated OPG at >1mg/ml is allowed to undergo air oxidation by dialysis in slightly basic phosphate buffer. The covalent thioether OPG dimer was prepared  
20      by reacting the bis-maleimide crosslinker, N,N-bis(3-maleimidopropianyl)-2-hydroxy 1,3 propane with the thiolated OPG at >1mg/ml at a 0.6x molar ratio of crosslinker:OPG in phosphate buffer at pH 6.5.  
25      Similarly, the PEG dumbbells are produced by reaction of substoichiometric amounts of bis-maleimide PEG crosslinkers with thiolated OPG at >1mg/ml in phosphate buffer at pH 6.5. Any of the above dimeric conjugates may be further purified using either ion exchange or size exclusion chromatographies.

30      Dimeric PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A prepared using the above procedures include disulfide-bonded OPG dimer, covalent thioether OPG dimer with an aliphatic amine type crosslinker, 3.4 kD and 8kD PEG dumbbells and monobells.

PEG-OPG conjugates were tested for activity in vitro using the osteoclast maturation assay described in Example 11A and for activity in vivo by measuring increased bone density after injection into mice as 5 described in Example 11C. The in vivo activity is shown below in Table 2.

Table 2  
In vivo biological activity of Pegylated OPG

10	<u>OPG Construct</u>	<u>Increase in Tibial Bone Density</u>
	muOPG met [22-194]	-
	muOPG met [22-194] 5k PEG	+
	muOPG met [22-194] 20k PEG	+
15	huOPG met [22-194] P25A	-
	huOPG met [22-194] P25A 5k PEG	+
	huOPG met [22-194] P25A 20k PEG	+
	huOPG met [22-194] P25A 31k PEG	+
20	huOPG met [22-194] P25A 57k PEG	+
	huOPG met [22-194] P25A 12k PEG	+
	huOPG met [22-194] P25A 20k Branched PEG	+
	huOPG met [22-194] P25A 8k PEG dimer	+
	<u>huOPG met [22-194] P25A disulfide crosslink</u>	+

25	<u>EXAMPLE 13</u>
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Effects of OPG-Fc during the course of Adjuvant  
Arthritis in Lewis rats

The aim of these studies is to investigate whether CHO produced OPG-Fc protects against adjuvant 30 arthritis-associated bone mineral density loss in male Lewis rats.

Animals

Male Lewis rats (Charles River, Wilmington MA) 8-9 weeks of age (n = 6) at the time of mycobacteria in oil 35 injection, were used. Two rats were housed per cage in an air conditioned environment (room temperature 23 ± 2

C, relative humidity 50 ± 20%) that illuminated from 6:30 am to 6:30 p.m. Animals were fed a commercial rodent chow (#8640, Tek Lab, Madison WI); calcium and phosphorus contents were 1.2% and 1.0%, respectively.

- 5 All animals were sacrificed by carbon dioxide inhalation.

Induction and Measurement of Adjuvant Arthritis

Adjuvant arthritis (Ada) was induced by a single injection of a suspension of Mycobacterium tuberculosis (Difco Laboratories, Detroit MI) in paraffin oil (Crescent Chemical Co., Hauppauge, NY). Mycobacteria were grounded in a mortar to fine powder and suspended in paraffin oil (10 mg/ml). The suspension was dispersed evenly just before injection of 0.05ml at the base of tail. Severity of inflammation was monitored by measuring the volume of hindpaws using volume displacement technique. The extent of inflammation was calculated as increase in paw volume compared to Day 0. In addition, body weight was measured daily.

- 20 OPG treatment and DEXA bone mass measurement

Male Lewis (normal and adjuvant-induced) rats received varying doses of OPG-FC (22-194) by subcutaneous daily injection (See graphs below for dosing) from day 9 to day 15. At the end of the experiment (day 16) bone mass measurement (DexaScans) of the tibiotarsal region was performed with a Hologic QDR 4500 dual-energy x-ray absorptiometer.

Statistical Analysis

- All results were expressed as the mean ± standard deviation of the mean. The p value of 0.05 was used in the calculation to determine whether there were any significant differences between any two groups. Statistical significance of difference was assessed by analysis of variance based on a Mann Whitney U test 35 using Statsoft software (Statsoft, Tulsa, OK).

ResultsOPG-Fc inhibits loss of Bone Mineral Density in adjuvant arthritis

To study the effects of OPG-Fc on BMD in adjuvant arthritis, paws from two experiments were analyzed by DEXA. The results of BMD measurements on the tibiotarsal region are shown in Figures 2 and 4. Bone protective effects were observed in rats with adjuvant-arthritis treated with OPG-Fc via subcutaneous daily injection (from day 9 to day 15 after mycobacteria injection). Treatment with OPG-Fc at 4, 1, 0.25, 0.06, .016, and 0.004 mg/kg showed 100%, 100%, 100%, 86%, 22, and 22% inhibition of bone mineral density loss respectively. Treatment of the intermediate and high doses of OPG-Fc (4 - 0.06 mg/kg) showed a statistically significant difference in BMD when compared to the OPG placebo treated control group ( $P < 0.05$ ).

However, treatment with OPG-Fc (at all doses) had no statistically significant effect on the severity of inflammation (Figure 1 and 3, AUC) or loss of body weight (data on file).

Conclusion

In conclusion, the results demonstrate that OPG-Fc have great efficacy in preventing bone density loss in the tibiotarsal region in arthritic rats. The inhibitory effects of OPG-Fc against bone changes occurred without any anti-inflammatory actions.

EXAMPLE 14Combination treatment with OPG-Fc and sTNF-RI onAdjuvant Arthritis in Male Lewis Rats

Male Lewis rats were injected with 0.5 mg heat-killed Mycobacterium tuberculosis H37Ra in mineral oil at the base of the tail. Rats were monitored for paw swelling and weight loss. Arthritis (paw swelling) developed after about 10 days. Paw swelling was calculated daily relative to paw volume on day 9

(beginning of treatment) and the area under the curve (AUC) from day 9 to 15 is given in the graph (Figure 31A). On day 16 at the end of the experiment DexaScans of the rats were taken and the calcaneus was evaluated 5 for loss of bone mineral density (BMD) as shown in Figure 31B.

\* \* \*

While the invention has been described in what is considered to be its preferred embodiments, it is not 10 to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such 15 modifications and equivalents.

Claims

What is claimed is:

1. A method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with substances selected from the group consisting of TNF- $\alpha$  inhibitors; serine protease inhibitors; IL-1 inhibitors; IL-6 inhibitors; IL-8 inhibitors; IL-18 inhibitors; ICE modulators; FGF-1 to FGF-10; FGF modulators; PAF antagonists; MMP modulators; NOS modulators; modulators of glucocorticoid receptor; modulators of glutamate receptor; modulators of LPS levels; and noradrenaline and modulators and mimetics thereof.
2. The method of Claim 1, wherein the OPG protein is OPG-Fc.
3. The method of claim 1, wherein an IL-1 inhibitor and the OPG protein are administered.
4. The method of claim 3, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
5. The method of Claim 4, wherein the OPG protein comprises an Fc region.
6. The method of claim 1, wherein a TNF- $\alpha$  inhibitor and the OPG protein are administered.
7. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, or a fragment of sTNF-RI or sTNF-RII linked to an Fc region.
8. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG sTNFR-I.
9. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises a 2.6 kD sTNF-RI fragment.
10. The method of claim 9, wherein the sTNF-RI fragment comprises 30 kD PEG.

11. The method of claim 6, wherein the TNF- $\alpha$  inhibitor comprises sTNF-RII linked to an Fc region.
12. The method of claim 6, wherein the TNF- $\alpha$  inhibitor is etanercept.
13. The method of Claim 10, wherein the OPG protein is OPG-Fc.
14. The method of claim 1, wherein a serine protease inhibitor and the OPG protein are administered.
15. The method of claim 14, wherein the serine protease inhibitor comprises a SLPI polypeptide.
16. The method of Claim 14, wherein the OPG protein is OPG-Fc.
17. The method of claim 1, wherein an IL-1 inhibitor, a TNF- $\alpha$  inhibitor, and the OPG protein are administered.
18. The method of claim 17, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
19. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR-Fc.
20. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG-sTNFR-I.
21. The method of claim 17, wherein the sTNF-RI fragment is a 2.6 kD fragment.
22. The method of claim 21, wherein the sTNF-RI fragment comprises 30 kD PEG.
23. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-II linked to an Fc region.
24. The method of claim 17, wherein the TNF- $\alpha$  inhibitor is etanercept.
25. The method of Claim 17, wherein the OPG protein is OPG-Fc.

26. The method of Claim 1, wherein an IL-1 inhibitor, a serine protease inhibitor, and the OPG protein are administered.
27. The method of claim 26, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
28. The method of claim 26, wherein the serine protease inhibitor comprises a SLPI polypeptide.
29. The method of Claim 26, wherein the OPG protein is OPG-FC.
30. The method of claim 1, wherein a serine protease inhibitor, a TNF- $\alpha$  inhibitor, and the OPG protein are administered.
31. The method of claim 30, wherein the serine protease inhibitor comprises a SLPI polypeptide.
32. The method of claim 17, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR-FC.
33. The method of claim 30, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG sTNFR-I.
34. The method of claim 30, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-II linked to an Fc region.
35. The method of claim 30, wherein the TNF- $\alpha$  inhibitor is etanercept.
36. The method of claim 30, wherein the TNF- $\alpha$  inhibitor comprises a 2.6 kD sTNF-RI fragment.
37. The method of claim 36, wherein the sTNF-RI fragment comprises 30 kD PEG.
38. The method of Claim 30, wherein the OPG protein is OPG-FC.
39. The method of any of claims 17 to 38, wherein the condition treated is rheumatoid arthritis.
40. The method of any of claims 17 to 38, wherein the condition treated is multiple sclerosis.

41. The method of any of claims 17 to 38, wherein the condition treated is osteoporosis.
42. The method of any of claims 17 to 38, wherein the condition treated is osteomyelitis.
43. A method of treating an IL-1 mediated disease, which comprises administering therapeutically effective amounts of an IL-1 inhibitor and a serine protease inhibitor.
44. The method of claim 43, wherein the IL-1 inhibitor comprises an IL-1ra polypeptide.
45. The method of claim 43, wherein the serine protease inhibitor comprises a SLPI polypeptide.
46. The method of claim 43, wherein the IL-1 mediated disease is asthma.
47. The method of claim 43, wherein the IL-1 mediated disease is rheumatoid arthritis.
48. The method of claim 46, wherein the therapeutically effective amounts are delivered by pulmonary administration.
49. A method of treating TNF-mediated disease, which comprises administering therapeutically effective amounts of a TNF- $\alpha$  inhibitor and a serine protease inhibitor.
50. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR-FC.
51. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises 30 kD PEG sTNFR-I.
52. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises a 2.6 kD STNF-RI fragment.
53. The method of claim 52, wherein the STNF-RI fragment comprises 30 kD PEG.

54. The method of claim 49, wherein the TNF- $\alpha$  inhibitor comprises sTNFR-II linked to an Fc region.
55. The method of claim 49, wherein the TNF- $\alpha$  inhibitor is etanercept.
56. The method of claim 49, wherein the serine protease inhibitor comprises a SLPI polypeptide.
57. The method of claim 49, wherein the TNF-mediated disease is rheumatoid arthritis.
58. A method of treating inflammation, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.
59. A method of treating rheumatoid arthritis, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.
60. A method of treating SLE, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.
61. A method of treating GvHD, which comprises administering an IL-18 inhibitor, a TNF- $\alpha$  inhibitor, and an IL-1 inhibitor.

FIG. 1A

FIG. 1 B

FRI-1	69	YLHYDPETGRQLLCDKCAPGTYLQHQH.	TVRRKTLCV.	PCPDY.	SYTDSSW
TNFR profile	6	YHYDQNQGRMCEECHMCQPGHFLVKHCKQPKRDTVCHKFCEPGVFTYTTDDW			
FRI-1	116	H			
TNFR profile	56	H			
			Z Score = 8.29		

EIC.

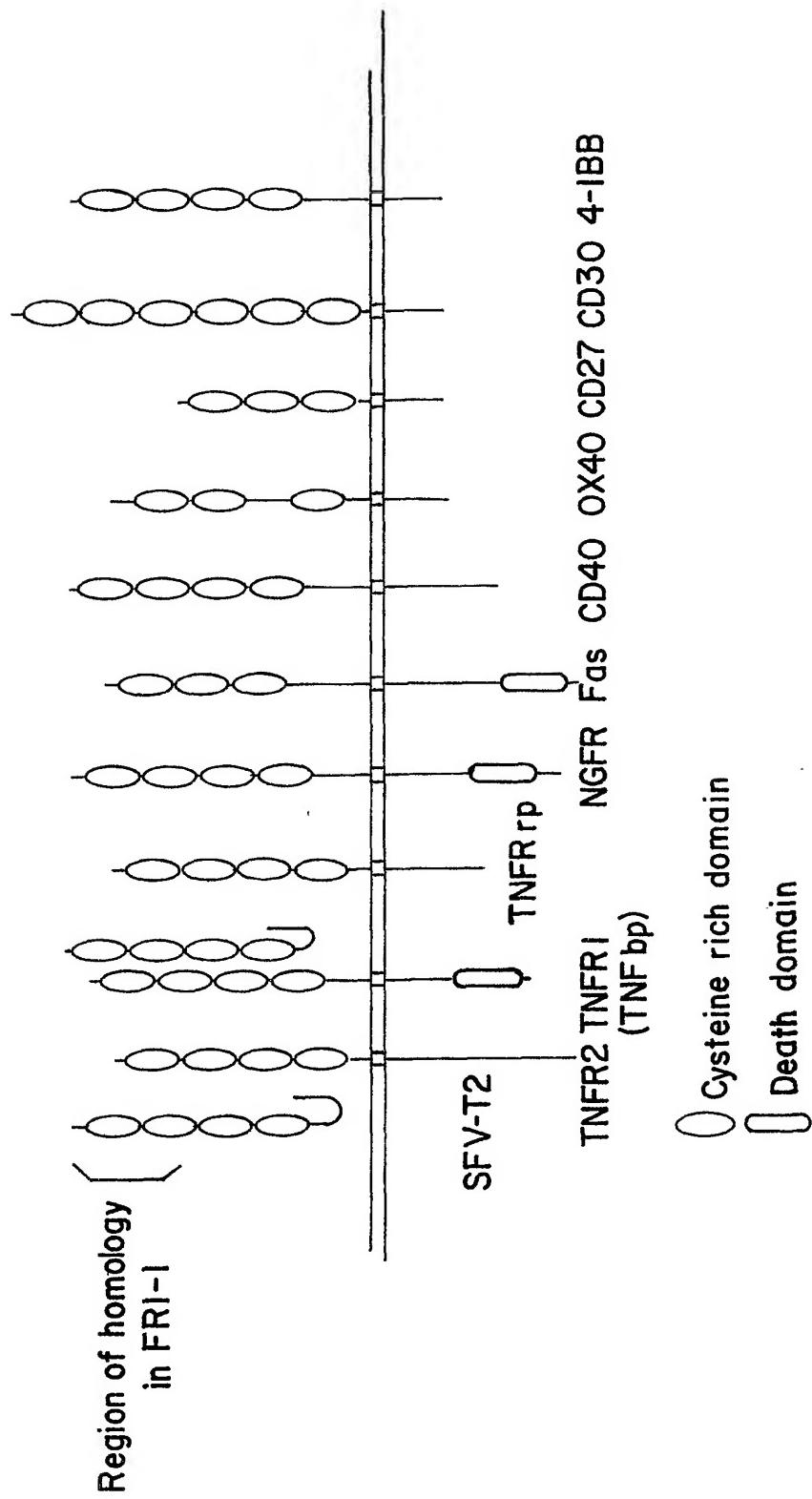


FIG.2A

AUG

TAG

SP

FIG.2B

# FIG.2C

1030	1050	1070
GAGAGCTTGCCTGGGAAGAAGATCAGCCCAGACCGAGATTGAGAGAACGAGAAAGACCTGC		
E S L P G K K I S P D E I E R T R K T C		
1090	1110	1130
AAACCCAGCGAGCAGCTCCTGAAGCTACTGAGCTTGTGGAGGATCAAAAATGGAGACCAA		
K P S E Q L L K L L S L W R I K N G D Q		
1150	1170	1190
GACACCTTGAAGGGCCTGATGTACGCCTCAAGCACTTGAAAGCATACCACTTTCCAAA		
D T L K G L M Y A L K H L K A Y H F P K		
1210	1230	1250
ACCGTCACCCACAGTCTGAGGAAGACCATTAGGTTCTGCACAGCTTCACCATGTACCGA		
T V T H S L R K T I R F L H S F T M Y R		
1270	1290	1310
TTGTATCAGAAACTCTTCTAGAAATGATAGGAAATCAGGTTCAATCAGTGAAGATAAGC		
L Y Q K L F L E M I G N Q V Q S V K I S		
1330	1350	1370
TGCTTATAGTTAGGAATGGTCACTGGGCTGTTCTTCAGGATGGCCAACACTGATGGAG		
C L		
1390	1410	1430
CAGATGGCTGCTTCTCCGGCTCTGAAATGGCAGTTGATTCTTCATCAGTTGGTGG		
1450	1470	1490
GAATGAAGATCCTCCAGCCAACACACACACTGGGAGTCTGAGTCAGGAGAGTGAGGCA		
1510	1530	1550
GGCTATTGATAATTGTGCAAAGCTGCCAGGTGTACACCTAGAAAGTCAAGCACCTGAG		
1570	1590	1610
AAAGAGGATATTTTATAACCTCAAACATAGGCCCTTCCTCCTCTCCTATGGATGAG		
1630	1650	1670
TAATCAGAAGGCTTCTACTATCTTCTGTGTCATCCCTAGATGAAGGCCTCTTTATTAT		
1690	1710	1730
TTTTTATTCTTTTCGGAGCTGGGACCGAACCCAGGGCCTGCGCTTGCAGGGCAA		
1750	1770	1790
GTGCTCTACCACTGAGCTAAATCTCCAACCCCTGAAGGCCTCTTCTGCCTCTGAT		
1810	1830	1850
AGTCTATGACATTCTTTCTACAATTGCTACAGGTGCACGAGCCTATCCCATTGT		
1870	1890	1910
AGGTTCTAGGCAAGTTGACCGTTAGCTATTTCCCTCTGAAGATTGATTGAGTTGC		
1930	1950	1970
AGACTTGGCTAGACAAGCAGGGTAGTTATGGTAGTTATTAACAGACTGCCACCAGG		
1990	2010	2030
AGTCCAGTGTCTTCTGTTCTGTAGTTGACTTAAGCTGACTCCAAGTACATTAGTA		
2050	2070	2090
TGAAAAATAATCAACAAATTATTCTTCTATCAACATTGGCTAGCTTGTTCAGGGC		
2110	2130	2150
ACTAAAAGAAACTACTATATGGAGAAAGAATTGATATTGCCCAACGTTCAACAACCCA		
2170	2190	2210
ATAGTTATCCAGCTGTATGCCCTGGTCAGTGTCTACTGACTATGCCCTTATTAC		
2230	2250	2270
TGCATGCAGTAATTCAACTGGAAATAGTAATAATAATAGAAATAAAATCTAGACTCC		
2290	2310	2330
ATTGGATCTCTGAATATGGGAATATCTAAGTAAAGAAGCTTGTGAGATTTCAGTTGTGT		
2350	2370	2390
TAAAGGCTTTATTAAAAAGCTGATGCTTCTGTAAAAGTTACTAATATCTGTAAGA		
2410	2430	
CTATTACAGTATTGCTATTTATCCATCCAG		

FIG. 2D

fas.frg	M L G I W T - - -	- L L P L V L T S -	V A R L S S K S V N A Q V T D I N S	K G D R E K R D S V C	45	44
tnfr1.frg	- M G L S T V P D -	- L L P L V L T S -	V A R L S S K S V N A Q V T D I N S	K G D R E K R D S V C	25	25
sfv-t2.frg	- - - - -	- - - - -	- - - - -	- - - - -	39	39
sfv-t2.frg	- - - - -	- - - - -	- - - - -	- - - - -	28	28
tnfr2.frg	- - - - -	- - - - -	- - - - -	- - - - -	26	26
cd40.frg	- - - - -	- - - - -	- - - - -	- - - - -	34	34
osteо.frg	- - - - -	- - - - -	- - - - -	- - - - -	28	28
ngfr.frg	- - - - -	- - - - -	- - - - -	- - - - -	25	25
ox40.frg	- - - - -	- - - - -	- - - - -	- - - - -	-	-
41bb.frg	- - - - -	- - - - -	- - - - -	- - - - -	-	-
fas.frg	M L G I W T - - -	- L L P L V L T S -	V A R L S S K S V N A Q V T D I N S	K G D R E K R D S V C	95	94
tnfr1.frg	- M G L S T V P D -	- L L P L V L T S -	V A R L S S K S V N A Q V T D I N S	K G D R E K R D S V C	74	74
sfv-t2.frg	- - - - -	- - - - -	- - - - -	- - - - -	88	88
tnfr2.frg	- - - - -	- - - - -	- - - - -	- - - - -	88	88
cd40.frg	- - - - -	- - - - -	- - - - -	- - - - -	72	72
osteо.frg	- - - - -	- - - - -	- - - - -	- - - - -	75	75
ngfr.frg	- - - - -	- - - - -	- - - - -	- - - - -	78	78
ox40.frg	- - - - -	- - - - -	- - - - -	- - - - -	72	72
41bb.frg	- - - - -	- - - - -	- - - - -	- - - - -	54	54
fas.frg	T Q N L E G L H D G Q F	I H P Q K D Q T A Q M C	S I C C C S K C O P C A P G T Y L K Q P C C G H G M V S R C D H T F C R K Y -	G K E Y T A S T N Q L W N S D Y T Q L W N D S W H S V T P C L D P G F Y N E A V N P V C K S C P S T F S S	- - - - -	- - - - -
tnfr1.frg	P Q G C G G R I R E Y Y D Q C -	C R I L D G Q C -	K Q Y L H D G Q C -	G F Y A K V H A N Q T V R D T V C H P C -	- - - - -	- - - - -
sfv-t2.frg	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
tnfr2.frg	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
cd40.frg	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
osteо.frg	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
ngfr.frg	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
ox40.frg	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
41bb.frg	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
fas.frg	H F - - - - -	- C L S K C V S C G S -	- C R K E M G Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N I F Q C E	- - - - -	103	144
tnfr1.frg	- H J R A - - -	- A P A C T V P E C T V P H Q C T V P C K Q C T Q C N H C R -	- - - - -	- - - - -	144	84
sfv-t2.frg	- H J R A - - -	- A P A C T V P E C T V P H Q C T V P C K Q C T Q C N H C R -	- - - - -	- - - - -	84	98
tnfr2.frg	- W - - - -	- V P E C T V P H Q C T V P C K Q C T Q C N H C R -	- - - - -	- - - - -	98	85
cd40.frg	- R E - - -	- I R C H Q C T V P C K Q C T Q C N H C R -	- - - - -	- - - - -	85	85
osteо.frg	- R E - - -	- I R C H Q C T V P C K Q C T Q C N H C R -	- - - - -	- - - - -	89	89
ngfr.frg	- A T E P C K Q C T Q C N H C R -	- - - - -	- - - - -	- - - - -	89	84
ox40.frg	- Y D T G Q P N C -	- - - - -	- - - - -	- - - - -	84	84
41bb.frg	- I G G Q P N C -	- - - - -	- - - - -	- - - - -	-	-

FIG. 2E

fas.frg	-	C R L C D E G H V E I N G L E V E I N G L E	G L E V E I N G L E	G L E V E I N G L E
tnf1r1.frg	-	N C S L C L N G -	- T V H L S Q P A C T	- T V H L S Q P A C T
sfv-t2.frg	-	- - - - -	- - - - -	- - - - -
tnf2.frg	-	- P C T S D O -	- S E T Q K E G C T A E	- S E T Q K E G C T A E
cd40.frg	-	- - - - -	- S D T V C R T H N R A V C O E	- S D T V C R T H N R A V C O E
osteo.frg	-	- - - - -	- N Q E L O T H N R A V C O E	- N Q E L O T H N R A V C O E
ngfr.frg	-	- - - - -	- V C K Q S M -	- V C K Q S M -
ox40.frg	-	- - - - -	- V G L Q S E L -	- V G L Q S E L -
41bb.frg	-	- - - - -	- R S G G S E Y E R F K K A G Y E R F K K A G Y E	- R S G G S E Y E R F K K A G Y E R F K K A G Y E

fas.frg	V W V - - -	- K R K E V O K T C R K E N Q S H E S S P T I N P E - - -	- - - - -
tnfr1.frg	G L M Y R Y Q R W K S K L Y S I V C G K S T P E K E K E T A - - -	- G H N E V I H P A N P L A P N P S E F T P G F T	- - - - -
sfv-t2.frg	P V N - - -	- - - - -	- - - - -
tnfr2.frg	A S R - - -	- - - - -	- - - - -
cd40.frg	Q K G - - -	- - - - -	- - - - -
osteo.frg	Q K G - - -	- - - - -	- - - - -
ngfr.frg	E C T R W A D A E C E E T P G R - - -	- - - - -	- - - - -
ox40.frg	H P A S N S V C E D R S I L A T L W E P S T T	- - - - -	- - - - -
41bb.frg	K T - - -	- - - - -	- - - - -

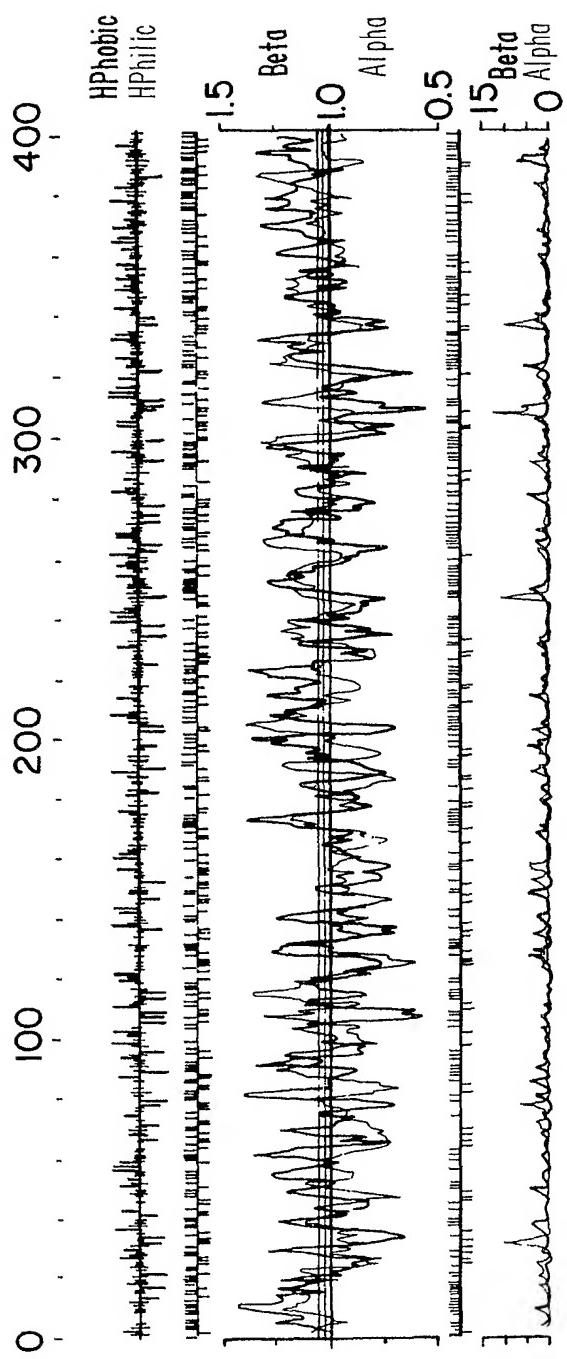


FIG. 3A  
FIG. 3B

FIG. 3C  
FIG. 3D  
FIG. 3E

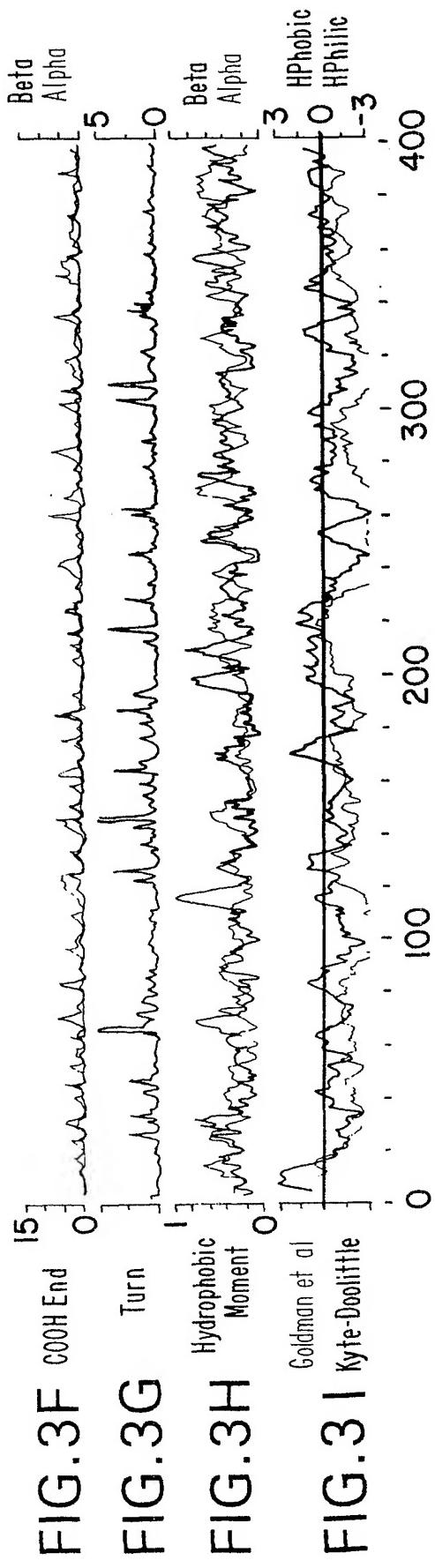


FIG.4A

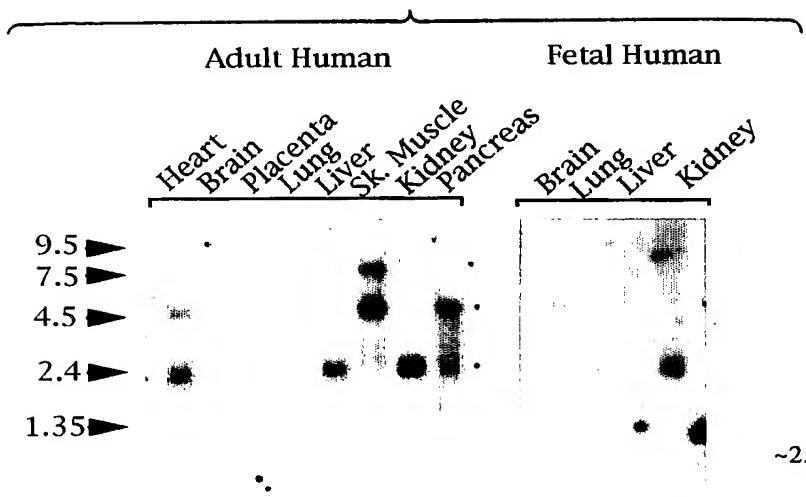
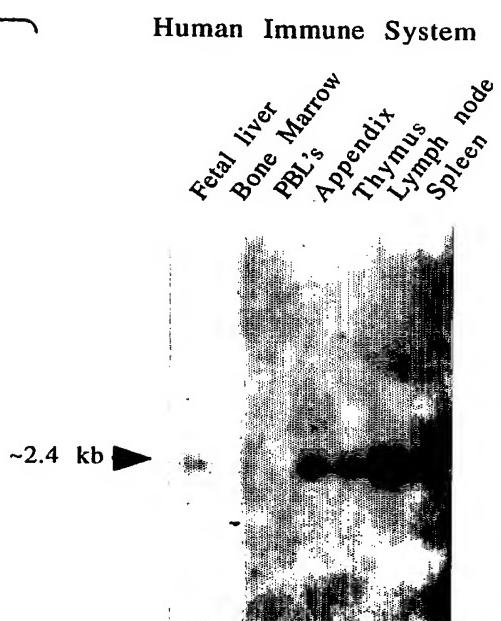
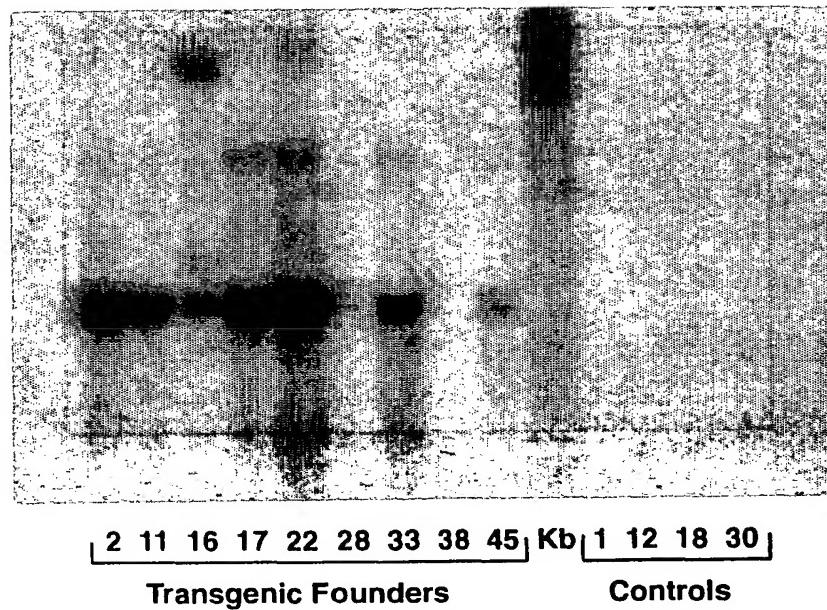
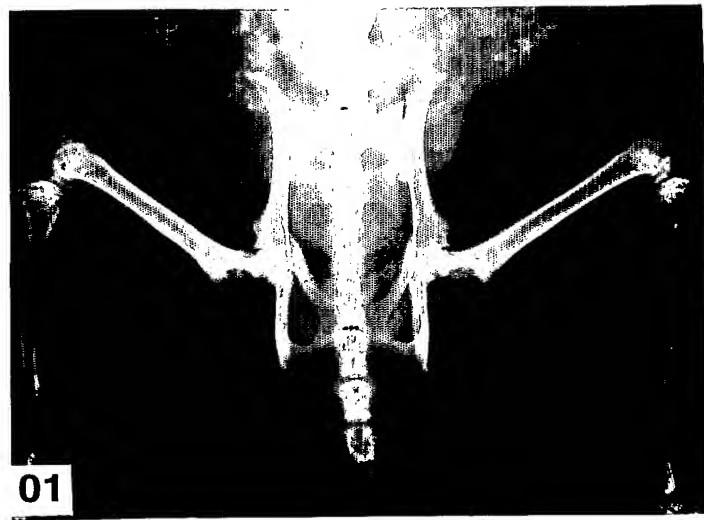
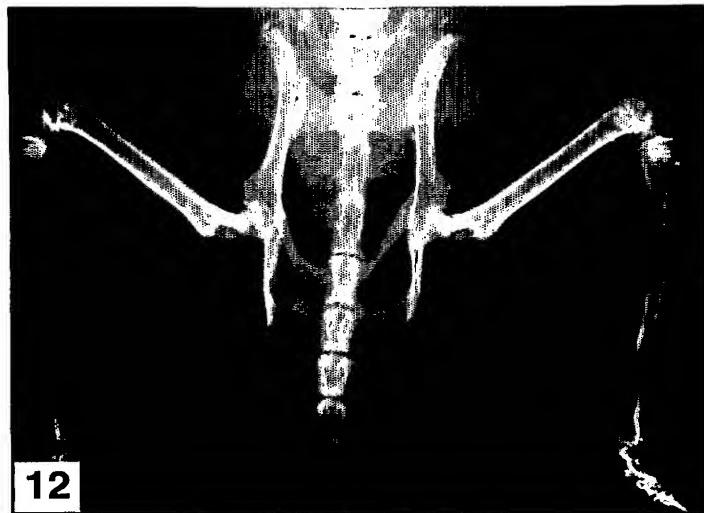
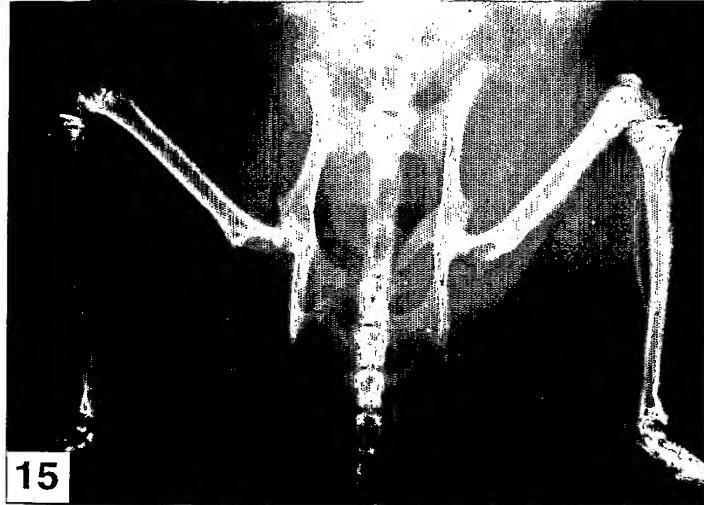
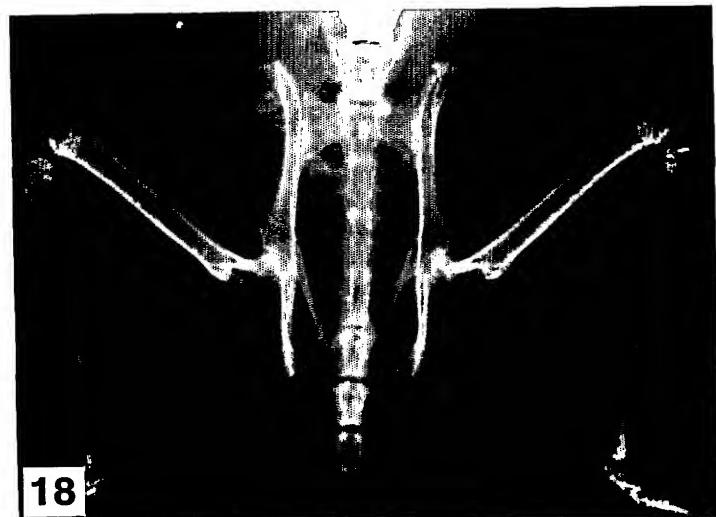
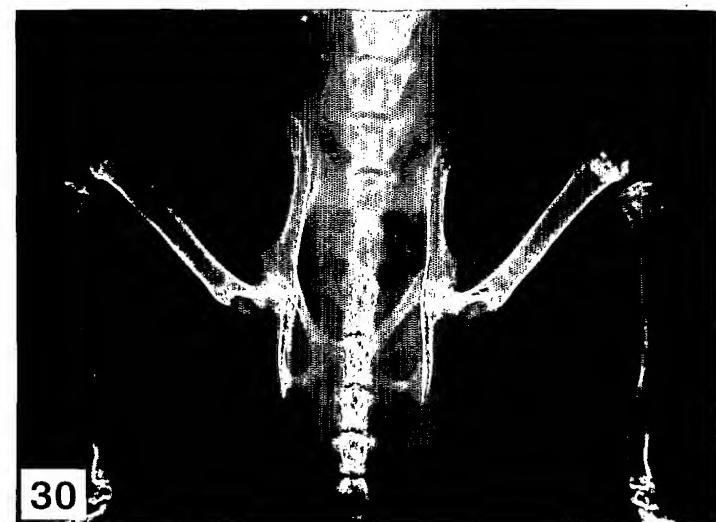


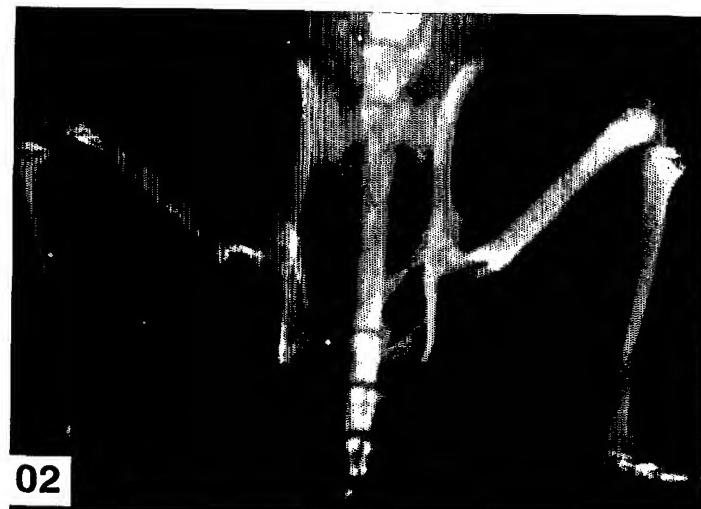
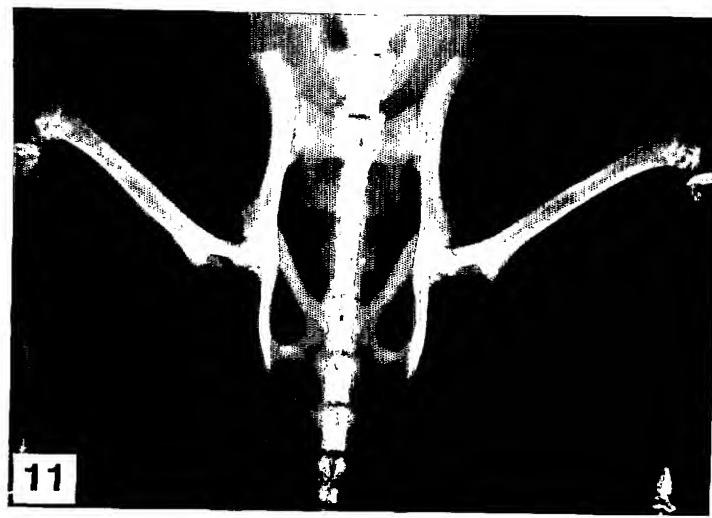
FIG.4B

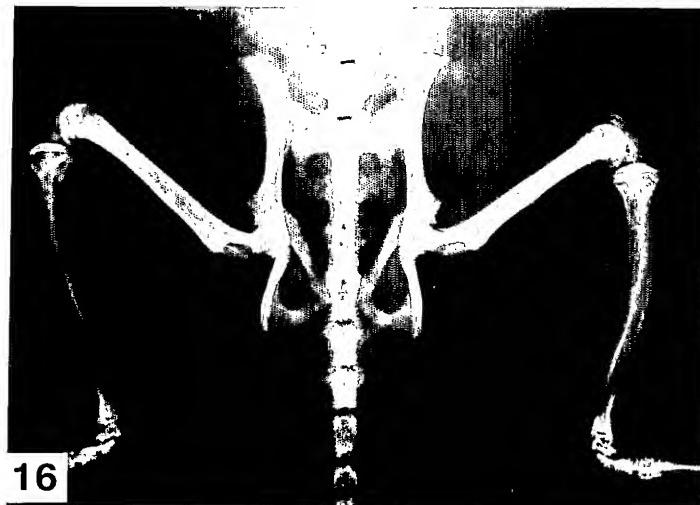
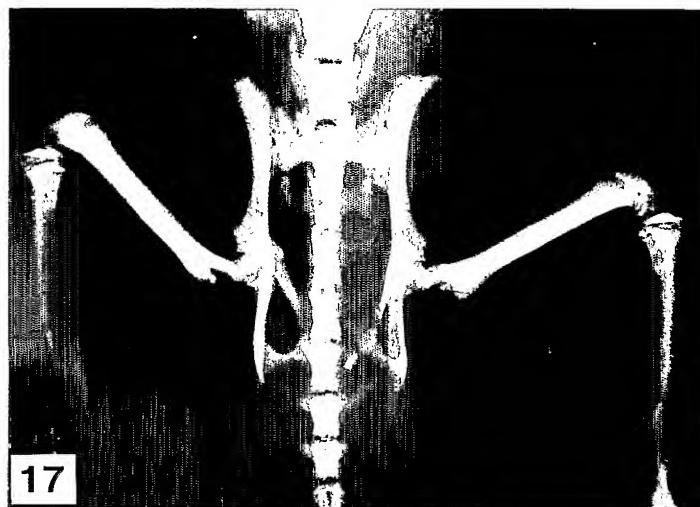


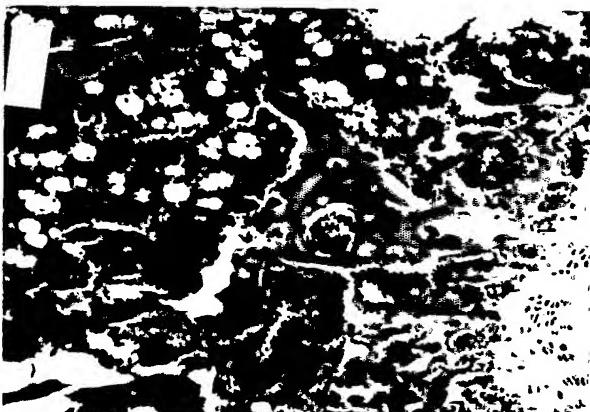
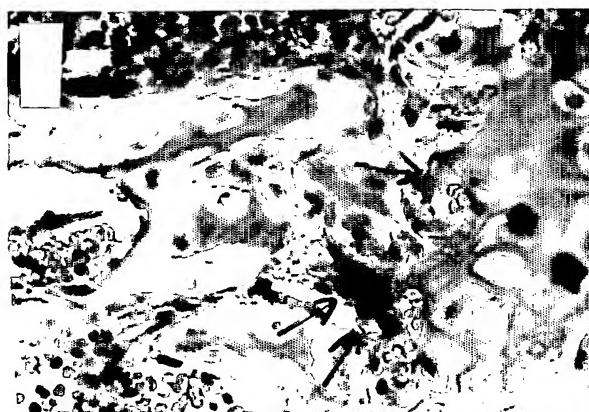
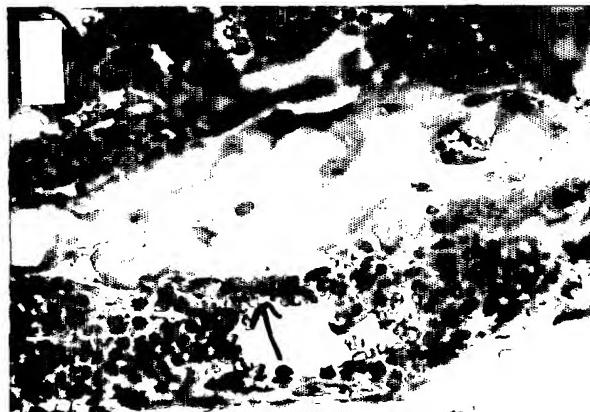
**FIG.5**

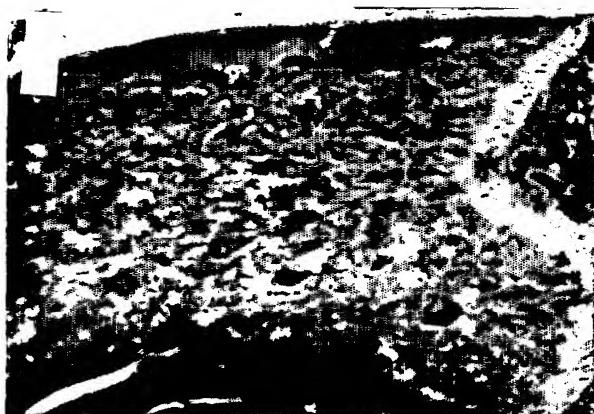
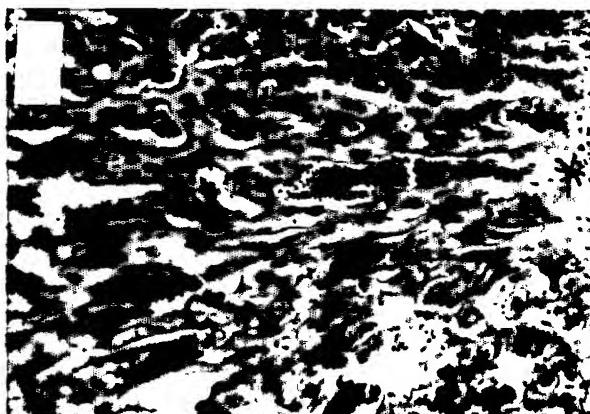
**FIG.6A****FIG.6B****FIG.6C**

**FIG.6D****FIG.6E****FIG.6F**

**FIG.6G****FIG.6H**

**FIG.6I****FIG.6J**

**FIG.7A****FIG.7B****FIG.7C****FIG.7D**

**FIG. 7E****FIG. 7F****FIG. 7G****FIG. 7H**

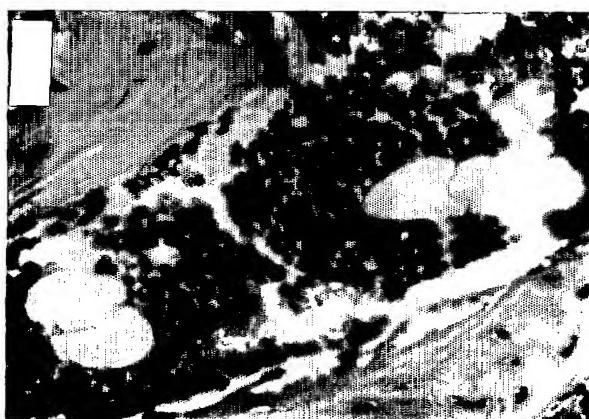
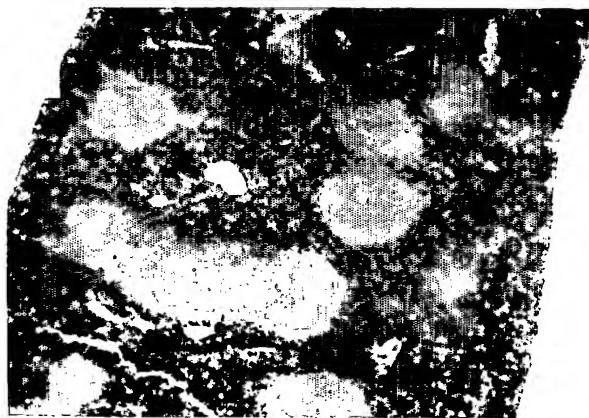
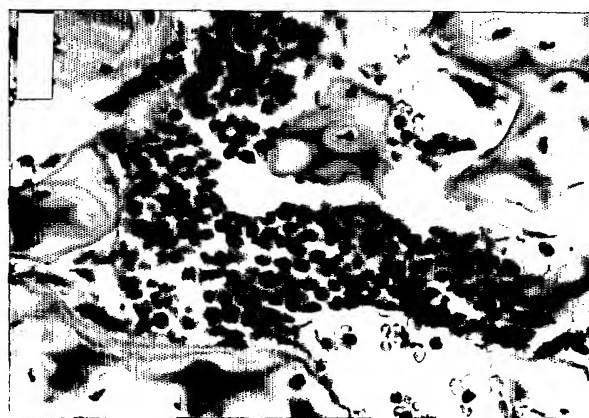
**FIG.8A****FIG.8B****FIG.8C****FIG.8D**

FIG.9A

# FIG. 9B

790	810	830
AAAGTGAATGCCGAGAGTGTAGAGAGGATAAAACGGAGACACAGCTCACAAAGAGCAAACC		
K V N A E S V E R I K R R H S S S Q E Q T		
850	870	890
TTCCAGCTGCTGAAGCTGTGGAAACATCAAAACAGAGACCAGGAAATGGTGAAGAAGATC		
F Q L L K L W K H Q N R D Q E M V K K I		
910	930	950
ATCCAAGACATTGACCTCTGTGAAAGCAGCGTCAGCGGCATCTCGGCCACTCGAACCTC		
I Q D I D L C E S S V Q R H L G H S N L		
970	990	1010
ACCACAGAGCAGCTTCTGCCTTGATGGAGAGCCTGCCTGGGAAGAAGATCAGCCCAGAA		
T T E Q L L A L M E S L P G K K I S P E		
1030	1050	1070
GAGATTGAGAGAACGAGAAAGACCTGCAAATCGAGCGAGCAGCTCCTGAAGCTACTCAGT		
E I E R T R K T C K S S E Q L L K L L S		
1090	1110	1130
TTATGGAGGATCAAAATGGTGACCAAGACACCTTGAAAGGGCCTGATGTATGCCCTCAAG		
L W R I K N G D Q D T L K G L M Y A L K		
1150	1170	1190
CACTTGAAAACATCCCACTTCCAAAATGTCACCCACAGTCTGAGGAAGACCATGAGG		
H L K T S H F P K T V T H S L R K T M R		
1210	1230	1250
TTCCTGCACAGCTTCACAATGTACAGACTGTATCAGAAGCTTTAGAAATGATAGGG		
F L H S F T M Y R L Y Q K L F L E M I G		
1270	1290	1310
AATCAGGTTCAATCCGTAAAATAAGCTGCTTATAACTAGGAATGGTCACTGGCTGTTT		
N Q V Q S V K I S C L		

CTTCA

FIG. 9C

10	30	50
GTATATATAACGTGATGAGCGTACGGGTGCGGAGACGCACCGGAGCGCTGCCAGCCGC		
70	90	110
CGYCTCCAAGCCCCTGAGGTTCCGGGACCACAATGAACAAGTTGCTGCTGCCGCT	M N K L L C C A L	
130	150	170
CGTGTTCCTGGACATCTCCATTAAAGTGGACCACCCAGGAAACGTTCCCTCAAAGTACCT	V F L D I S I K W T T Q E T F P P K Y L	
190	210	230
TCATTATGACGAAGAACCTCTCATCAGCTGTTGTGACAAATGTCCTCCTGGTACCTA	H Y D E E T S H Q L L C D K C P P G T Y	
250	270	290
CCTAAAACAACACTGTACAGCAAAGTGGAGACCGTGTGCGCCCCCTGCCCTGACCACTA	L K Q H C T A K W K T V C A P C P D H Y	
310	330	350
CTACACAGACAGCTGGCACACCAAGTGACGAGTGTCTATACTGCAGCCCCGTGTGCAAGGA	Y T D S W H T S D E C L Y C S P V C K E	
370	390	410
GCTGCAGTACGTCAAGCAGGAGTGCATCGCACCCACAACCGCGTGTGCGAATGCAAGGA	L Q Y V K Q E C N R T H N R V C E C K E	
430	450	470
AGGGCGCTACCTTGAGATAGAGTTCTGCTTGAACATAGGAGCTGCCCTGGATTGG	G R Y L E I E F C L K H R S C P P G F G	
490	510	530
AGTGGTGCAAGCTGGAACCCAGAGCGAAATACAGTTGCAAAAGATGTCAGATGGGTT	V V Q A G T P E R N T V C K R C P D G F	
550	570	590
CTTCTCAAATGAGACGTACATCTAAAGCACCCGTAGAAAACACACAAATTGCAGTGTCTT	F S N E T S S K A P C R K H T N C S V F	
610	630	650
TGGTCTCCTGCTAACTCAGAAAGGAAATGCAACACACGACAACATATGTTCCGGAAACAG	G L L L T Q K G N A T H D N I C S G N S	
670	690	710
TGAATCAACTAAAAATGTGGAATAGATGTTACCCGTGAGGAGGCATTCTCAGGTT	E S T Q K C G I D V T L C E E A F F R F	
730	750	770
TGCTGTTCCCTACAAAGTTACGCCCTAACTGGCTTAGTGTCTGGTAGACAATTGCCCTGG	A V P T K F T P N W L S V L V D N L P G	

## FIG.9D

790	810	830
CACCAAAGTAAACGCAGAGAGTGTAGAGAGGATAAAACGGCAACACAGCTCACAAAGAACAA		
T K V N A E S V E R I K R Q H S S S Q E Q		
850	870	890
GACTTCCAGCTGCTGAAGTTATGGAAACATCAAAACAAAGACCAAGATATAGTCAGAA		
T F Q L L K L W K H Q N K D Q D I V K K		
910	930	950
GATCATCCAAGATATTGACCTCTGTGAAAACAGCGTGCAGCGGCACATTGGACATGCTAA		
I I Q D I D L C E N S V Q R H I G H A N		
970	990	1010
CCTCACCTTCGAGCAGCTTCGTAGCTTGATGGAAAGCTTACCGGGAAAGAAAAGTGGGAGC		
L T F E Q L R S L M E S L P G K K V G A		
1030	1050	1070
AGAAGACATTGAAAAAACATAAAGGCATGCAAACCCAGTGACCAGATCCTGAAGCTGCT		
E D I E K T I K A C K P S D Q I L K L L		
1090	1110	1130
CAGTTTGTGGCGAATAAAAATGGCGACCAAGACACCTTGAAGGGCCTAATGCACGCACT		
S L W R I K N G D Q D T L K G L M H A L		
1150	1170	1190
AAAGCACTCAAAGACGTACCACTTCCCCTAAACTGTCACTCAGAGTCTAAAGAACCAT		
K H S K T Y H F P K T V T Q S L K K T I		
1210	1230	1250
CAGGTTCTTCACAGCTTCACAATGTACAAATTGTATCAGAAGTTATTTTAGAAATGAT		
R F L H S F T M Y K L Y Q K L F L E M I		
1270	1290	1310
AGGTAACCAGGTCCAATCAGAAAAATAAGCTGCTTATAACTGGAAATGCCATTGAGCT		
G N Q V Q S V K I S C L		
1330	1350	
GTTCCTCACAAATTGGCGAGATCCCATGGATGATAA		

## FIG. 9E

muosteо.frg	M N K W L C C A L L V L L D I I E W T T Q E T T P P K Y L H Y D P E T G H O L L C D K C A P G T Y L	50
ratosteо.frg	M N K W L C C A L L V F L D I I E W T T Q E T T P P K Y L H Y D P E T G R O L L C D K C A P G T Y L	50
huosteо.frg	M N K L I L C C A L V F L D I S I K W T T Q E T T P P K Y L H Y D E E T S H O L L C D K C P P G T Y L	50
muosteо.frg	K Q H C T V R R K T L C V P C P D H S Y T D S W H T S D E C V Y C S P V C K E L Q S V K Q E C N R T	100
ratosteо.frg	K Q H C T V R R K T L C V P C P D Y S Y T D S W H T S D E C V Y C S P V C K E L Q T V K Q E C N R T	100
huosteо.frg	K Q H C T A K W K T V C A P C P D H Y T D S W H T S D E C V Y C S P V C K E L Q Y V K Q E C N R T	100
muosteо.frg	H N R V C E C E E G R Y L E I E F C L K H R S C P P G S G V V Q A G T P E R N T V C K K C P D G F F	150
ratosteо.frg	H N R V C E C E E G R Y L E I E F C L K H R S C P P G L G V L Q A G T P E R N T V C K K R C P D G F F	150
huosteо.frg	H N R V C E C K E G R Y L E I E F C L K H R S C P P G F G V V Q A G T P E R N T V C K K R C P D G F F	150
muosteо.frg	S G E T S S K A P C I K H T N C S T I G L L I I Q G N A T H D N V C S G N R E A T Q K C G I D V T	200
ratosteо.frg	S G E T S S K A P C R K H T N C S S I G L L I I Q G N A T H D N V C S G N R E A T Q N C G I D V T	200
huosteо.frg	S N E T S S K A P C R K H T N C S S V F G L L I I Q G N A T H D N I C S G N S E S T Q K C G I D V T	200

## FIG. 9F

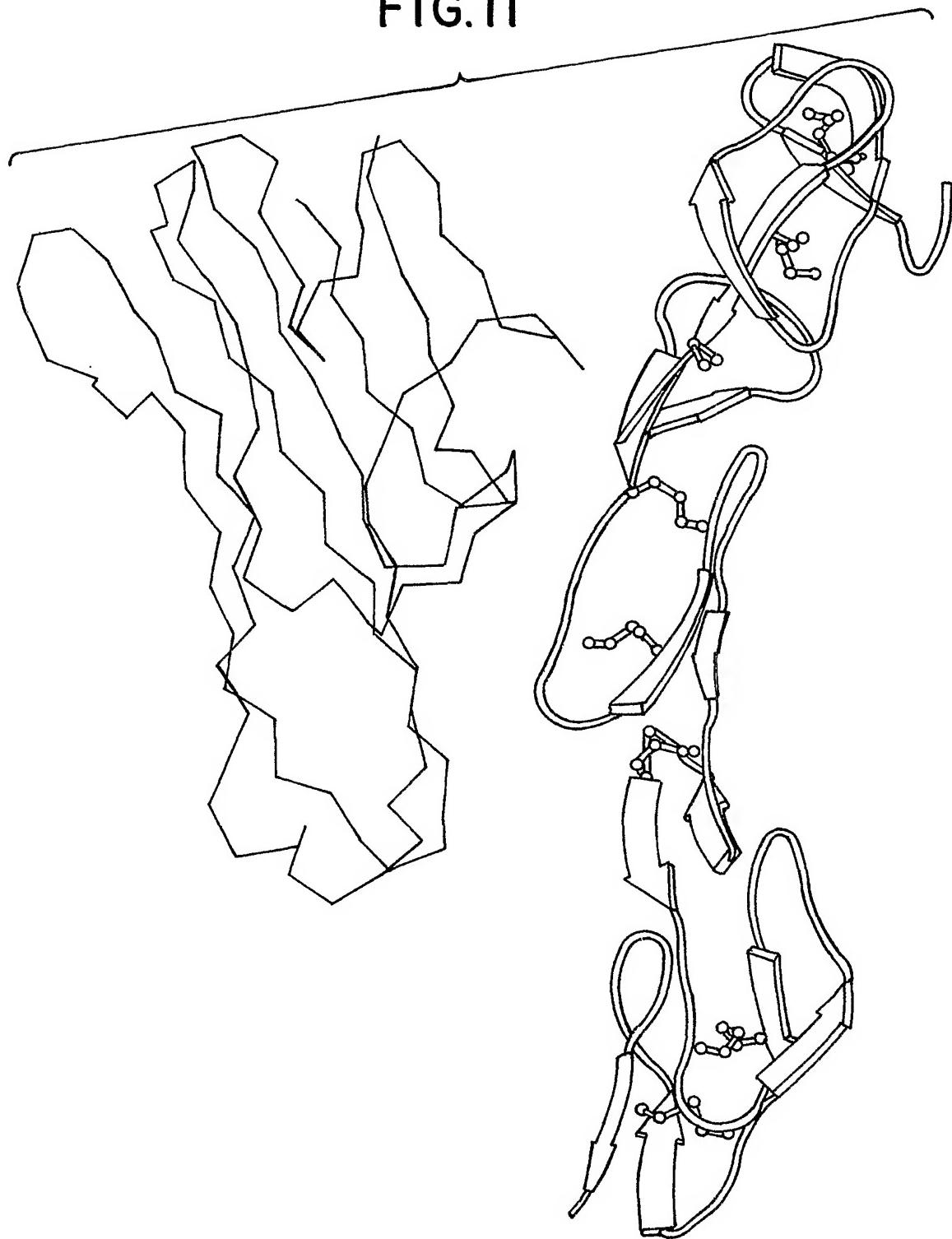
muosteо.frg	L C E E A F F R F A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R H S S Q E Q T	250
ratosteо.frg	L C E E A F F R F A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R H S S Q E Q T	250
huosteо.frg	L C E E A F F R F A V P T K I I P N W L S V L V D N I P G T K V N A E S V E R I K R Q H S S Q E Q T	250
muosteо.frg	F Q L K L W K H Q N R D Q E M V K I I Q D I D U C E S S V Q R H L G H S N L T T E Q L L A L M E	300
ratosteо.frg	F Q I L K L W K H Q N R D Q E M V K I I Q D I D U C E S S V Q R H I G H A N L T T E Q L R I L M E	300
huosteо.frg	F Q I L K L W K H Q N K D Q D I V K K I I Q D I D U C E N S V Q R H I G H A N L T T E Q L R S L M E	300
muosteо.frg	S L P G K K I S P E E I E R T R K T C K S S E Q L L K L S L W R I K N G D Q D T L K G L M Y A L K	350
ratosteо.frg	S L P G K K I S P D E I E R T R K T C K P S E Q L L K L S L W R I K N G D Q D T L K G L M Y A L K	350
huosteо.frg	S L P G K K V G A E D I E K T I K A C K P S D Q T I L K L L S L W R I K N G D Q D T L K G L M H A L K	350
muosteо.frg	H L K T S H F P K T V T H S S L R K T M R F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
ratosteо.frg	H L K A Y H F P K T V T H S L R K T I R F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
huosteо.frg	H S K T Y H F P K T V T Q S L K K T I R F L H S F T M Y K L Y Q K L F L E M I G N Q V Q S V K I S C	400
muosteо.frg	L	401
ratosteо.frg	L	401
huosteо.frg	L	401

## FIG. 10

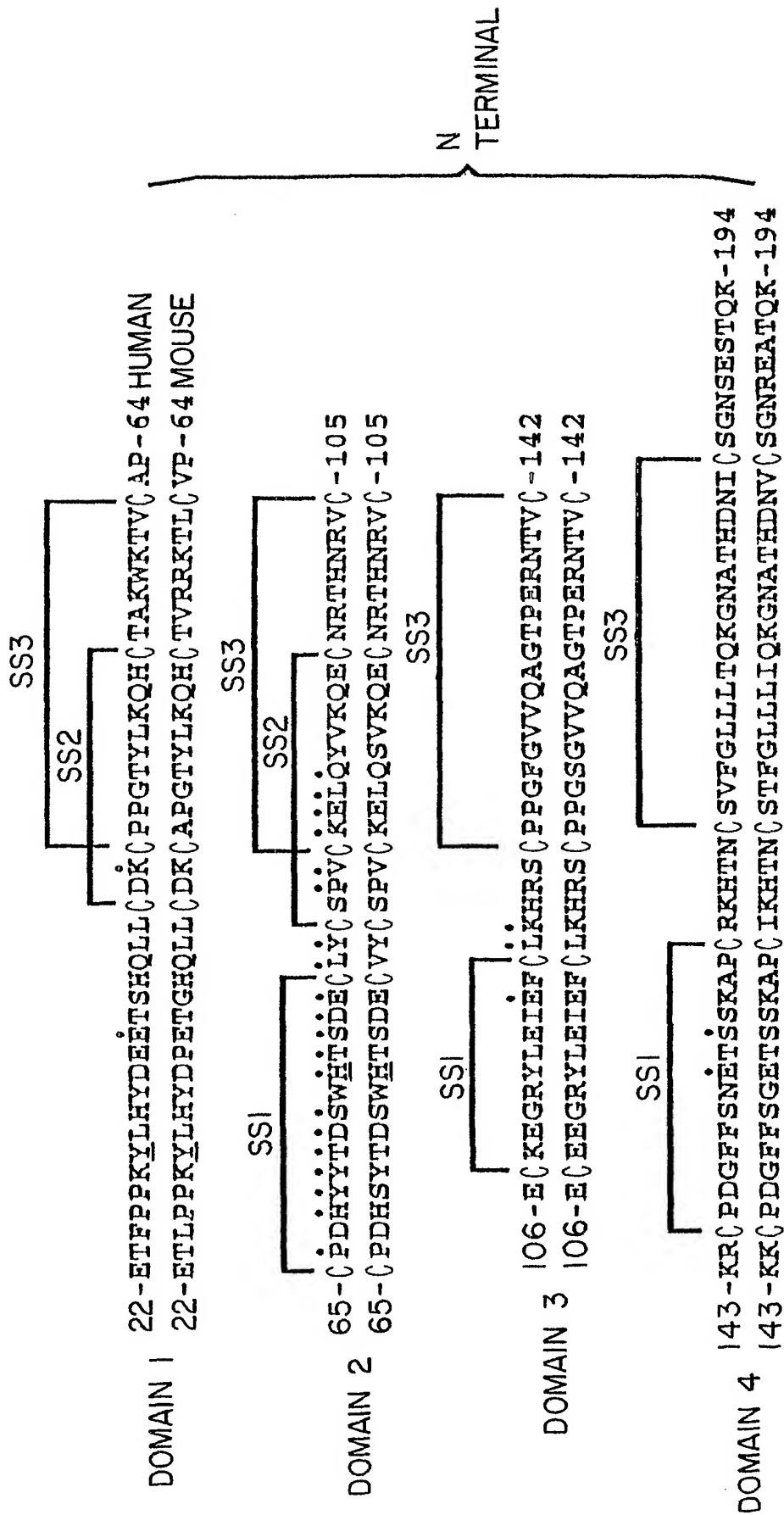
1tnrr C P Q - G K Y I H P O N N S I C C T K C H K G T Y L Y N D C P G P G Q D T D C R E C E S G S F T A S 49  
 humoste P P K Y L H Y D E E T S H Q L L C D K C P P G T Y L K Q H C T A K - W K T V C A P C P D H Y Y T D S 49.

1tnrr E N H L R H C L S C S - K C R K E M G Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F 98  
 humoste W H T S D E C L Y C S P V C - K E L Q Y V K - Q E C N R T H N R V C E C K E G R Y L E I - - E - F 93

1tnrr Q C F N C S L C L N G - T V H L S C Q E K Q N T V C T - C H A G F F L R E - - - N E C V S C 139  
 humoste - C L K H R S C P P G F G V V Q A G T P E R N T V C K R C P D G F F S N E T S S K A P C R K H 139

**FIG. II**

## FIG. 12A



## FIG. 12B

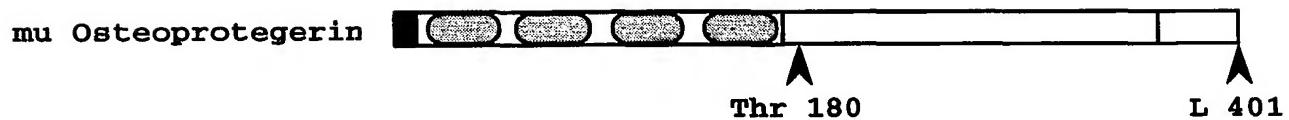
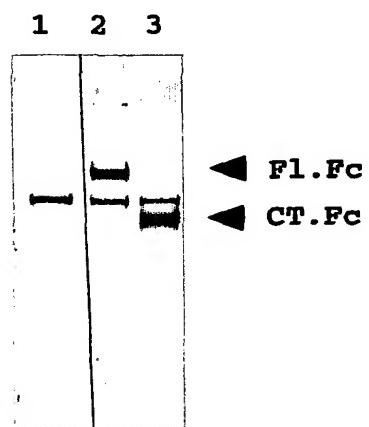
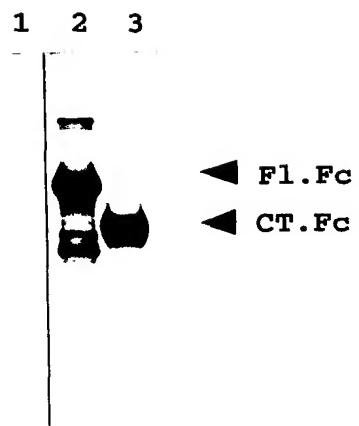
195 - C G I D V T L C E E A F F R A V P T K F T P N W L S V L V D N L P G T K V N A E S V E R I K R Q H S S - 2 4 6  
195 - C G I D V T L C E E A F F R A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R R H S S - 2 4 6

2 4 7 - Q E Q T E Q L L K L W K H Q N K D Q D I V K K I I Q D I D I C E N S V Q R H I G H A N L T F E Q L R S L - 2 9 8  
2 4 7 - Q E Q T F Q L L K L W K H Q N R D Q E M V R K K I I Q D I D I C E S S V Q R H L G H S N L T T E Q L L A L - 2 9 8

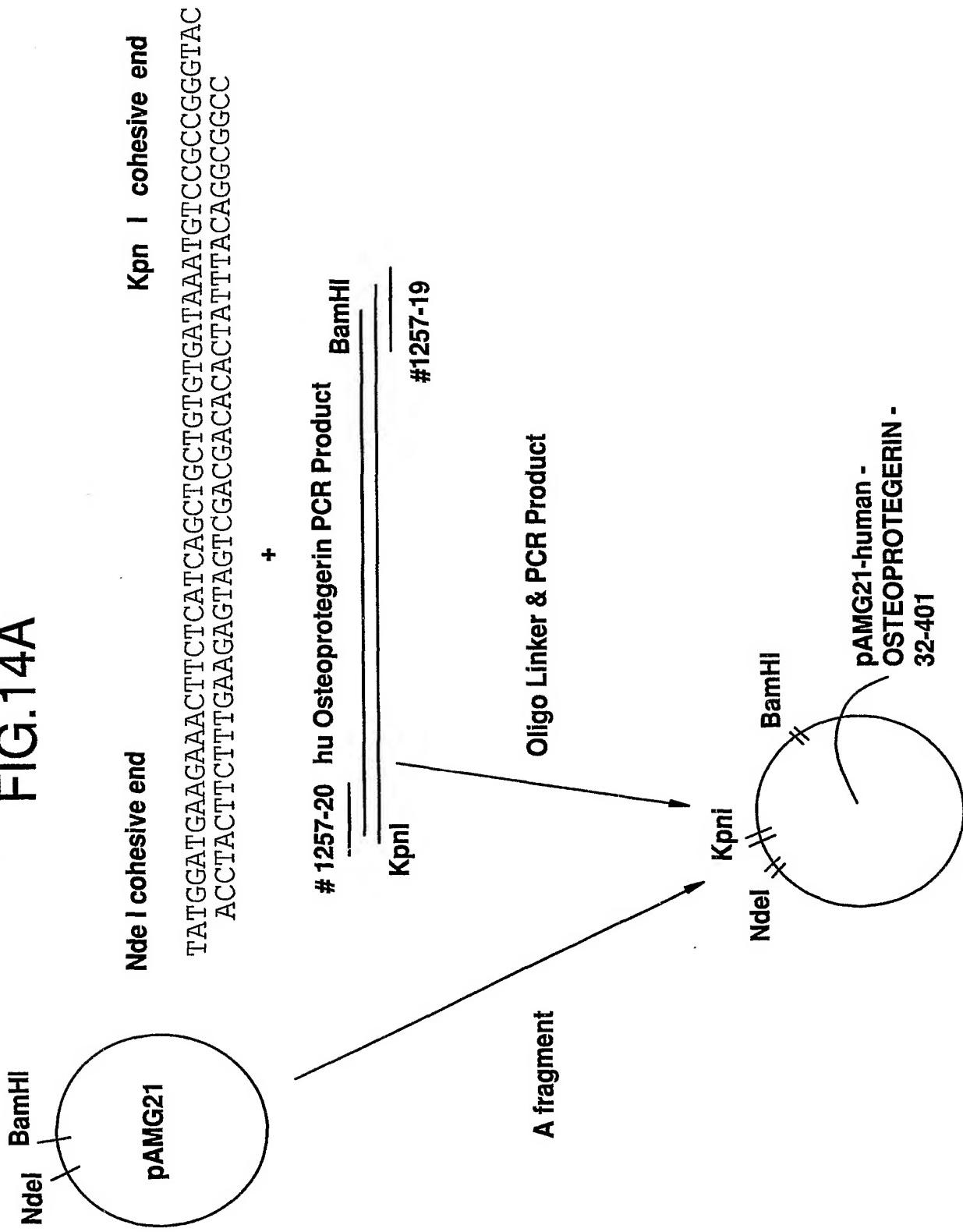
C TERMINAL

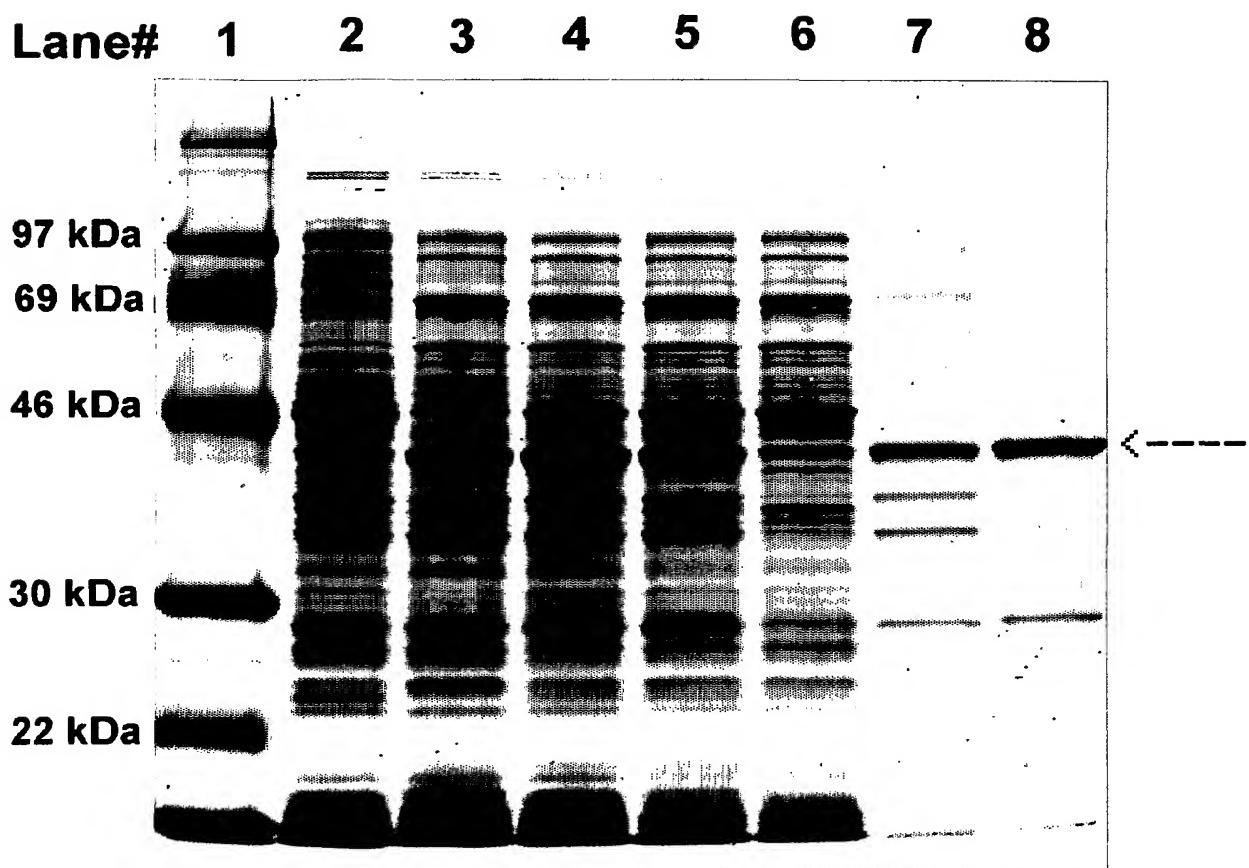
2 9 9 - M E S L P G K R V G A E D I E R T I R A C R P S D Q I L K L L S L W R I K N G D Q D T L R G L M H A L R - 3 5 0  
2 9 9 - M E S L P G K R I S P E E I E R T R K T C R S S E Q L K L L S L W R I K N G D Q D T L R G L M Y A L R - 3 5 0

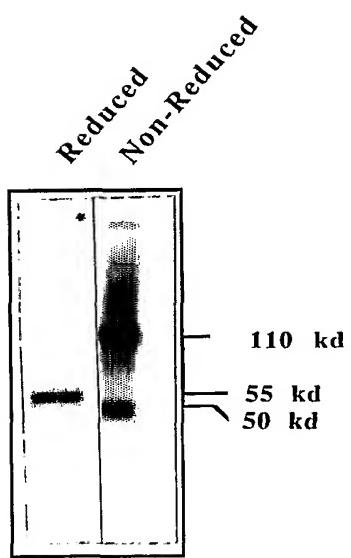
3 5 1 - H S K T Y H E P K T V T Q S L K K T I R F L H S F T M Y K L Y Q K L F L E M I G N Q V Q S V K I S C L - 4 0 1  
3 5 1 - H I K T S H E P K T V T H S L R K T M R F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C L - 4 0 1

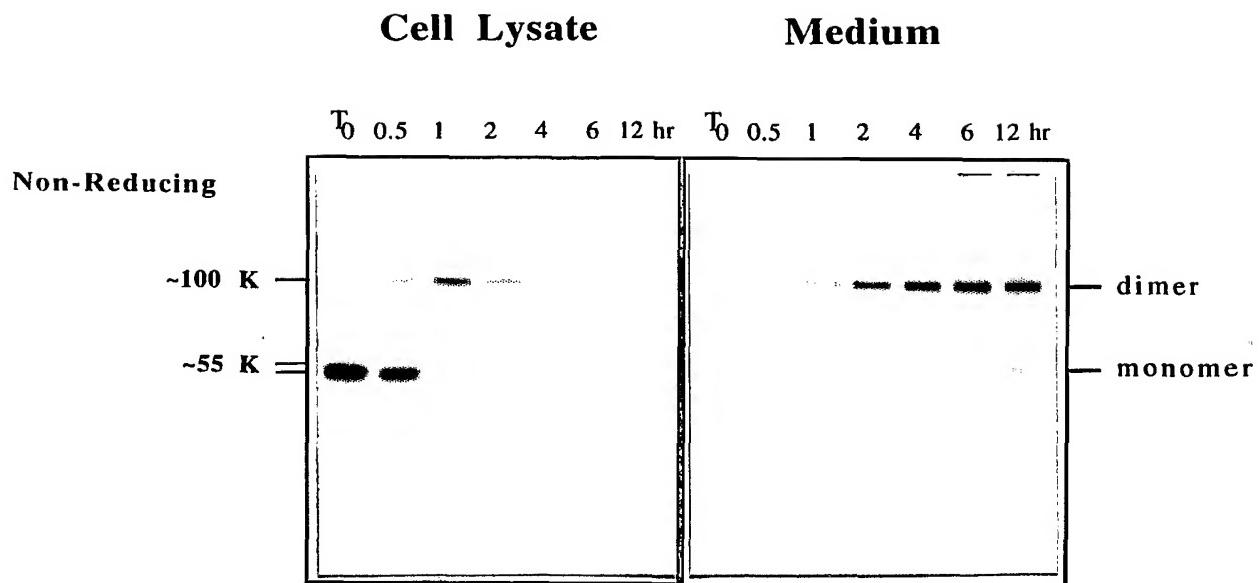
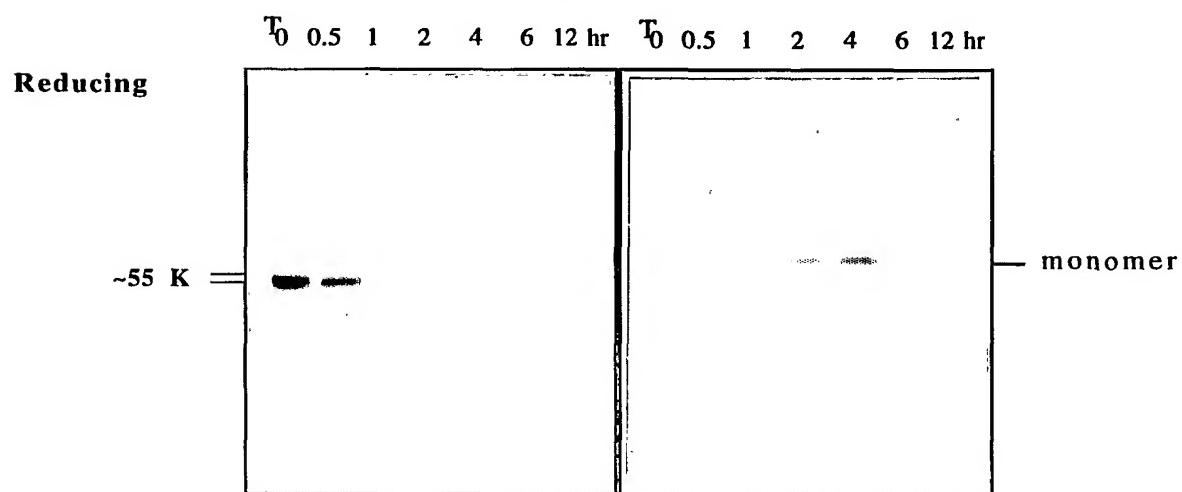
**FIG. 13A****FIG. 13B****FIG. 13C**

## FIG. 14A



**FIG. 14B**

**FIG. 15**

**FIG. 16A****FIG. 16B**

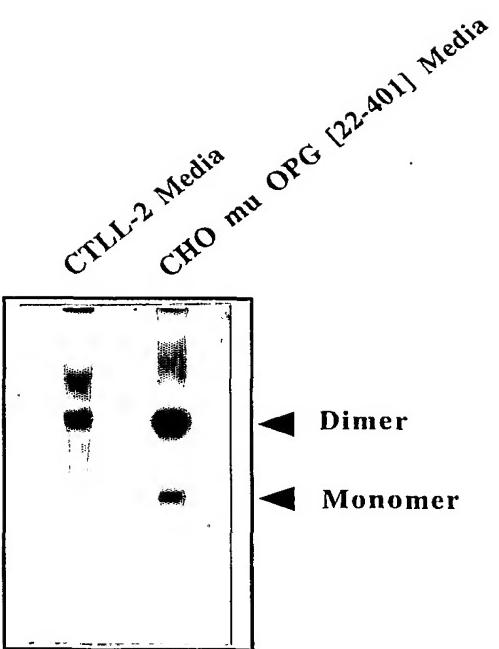
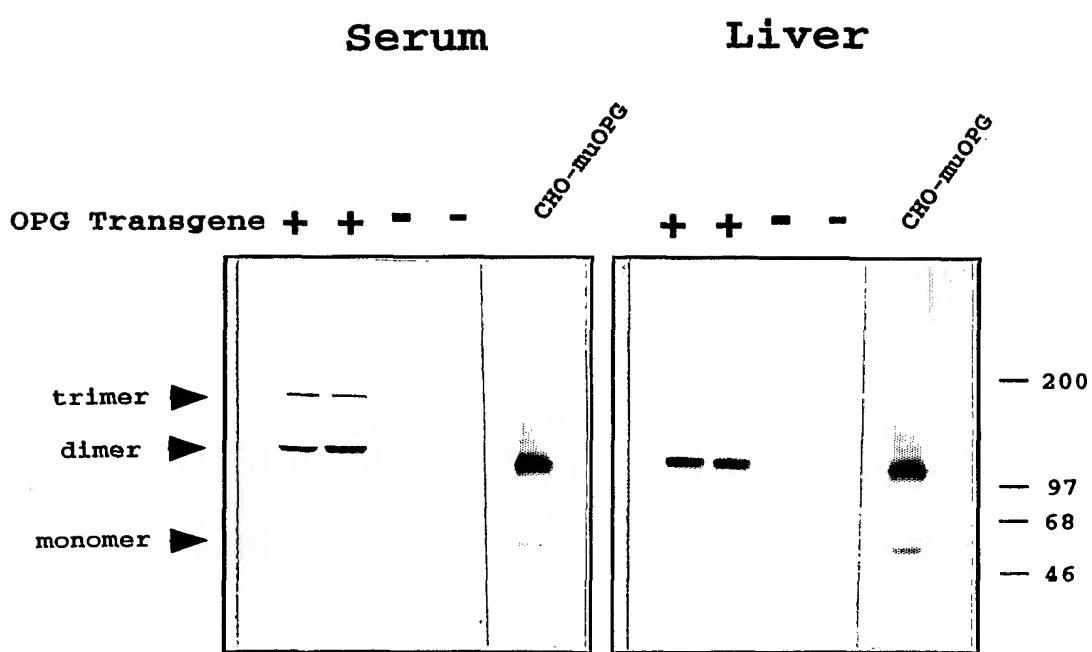
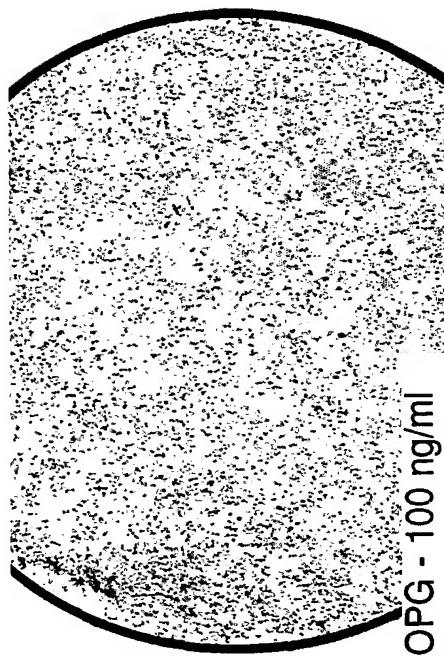
**FIG. 17**

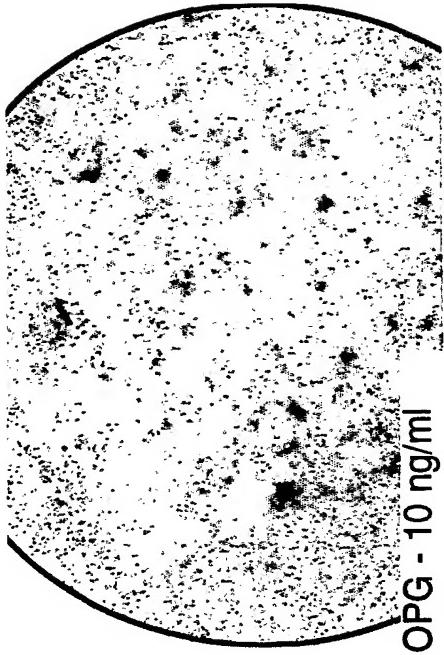
FIG. 18



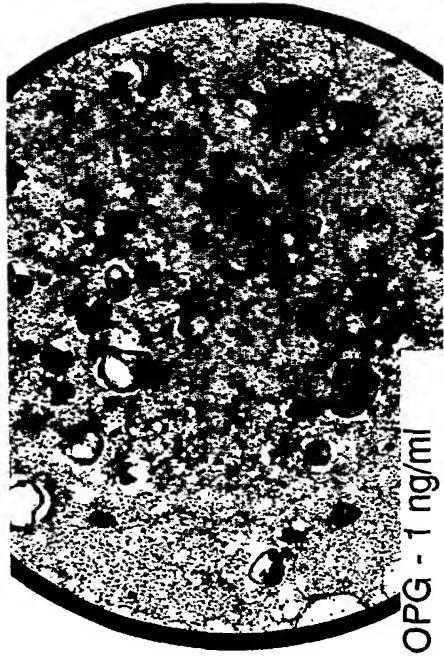
**FIG. 19A**



**FIG. 19B**



**FIG. 19C**



**FIG. 19D**

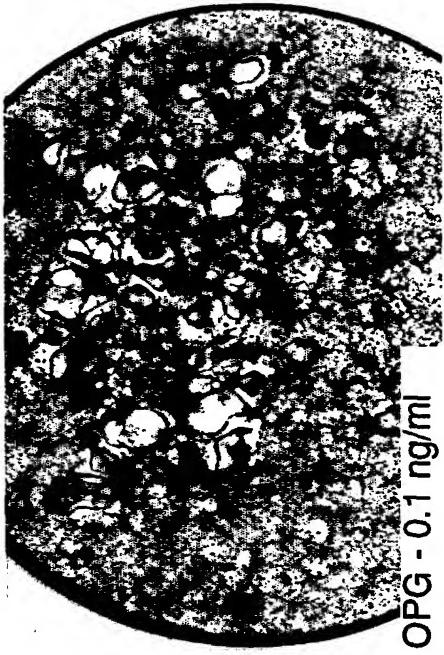
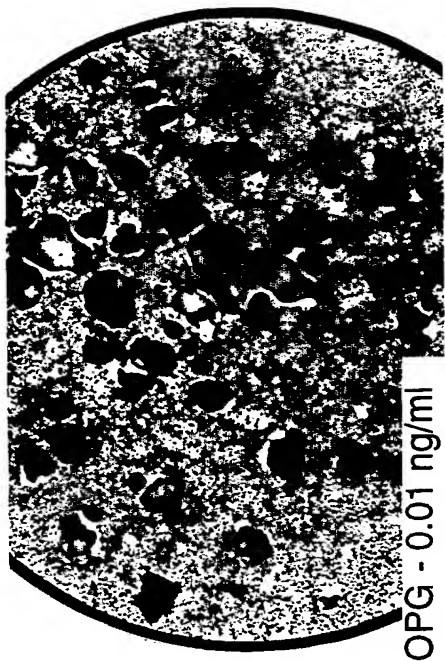
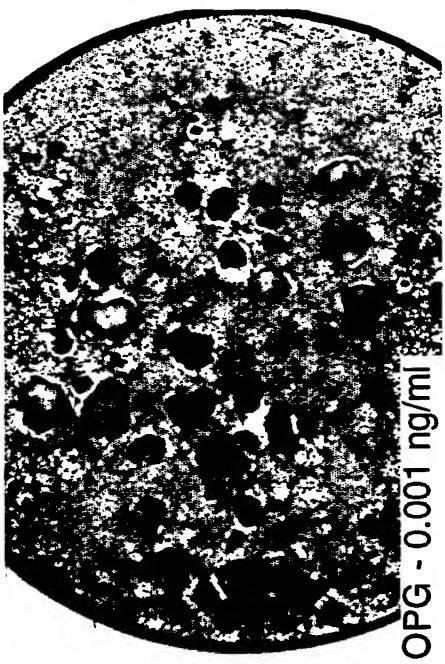


FIG. 19E



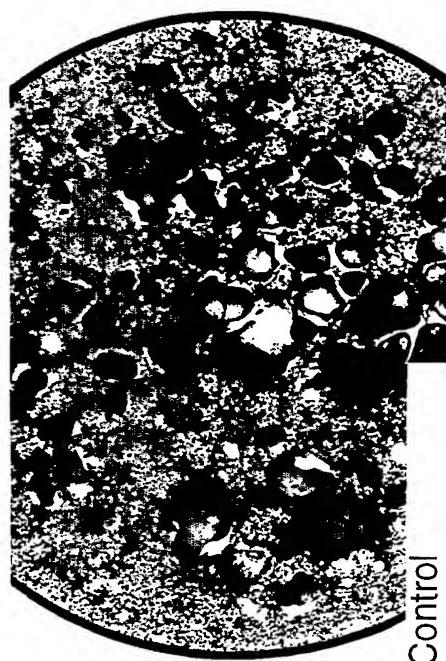
OPG - 0.01 ng/ml

FIG. 19F



OPG - 0.001 ng/ml

FIG. 19G



Control

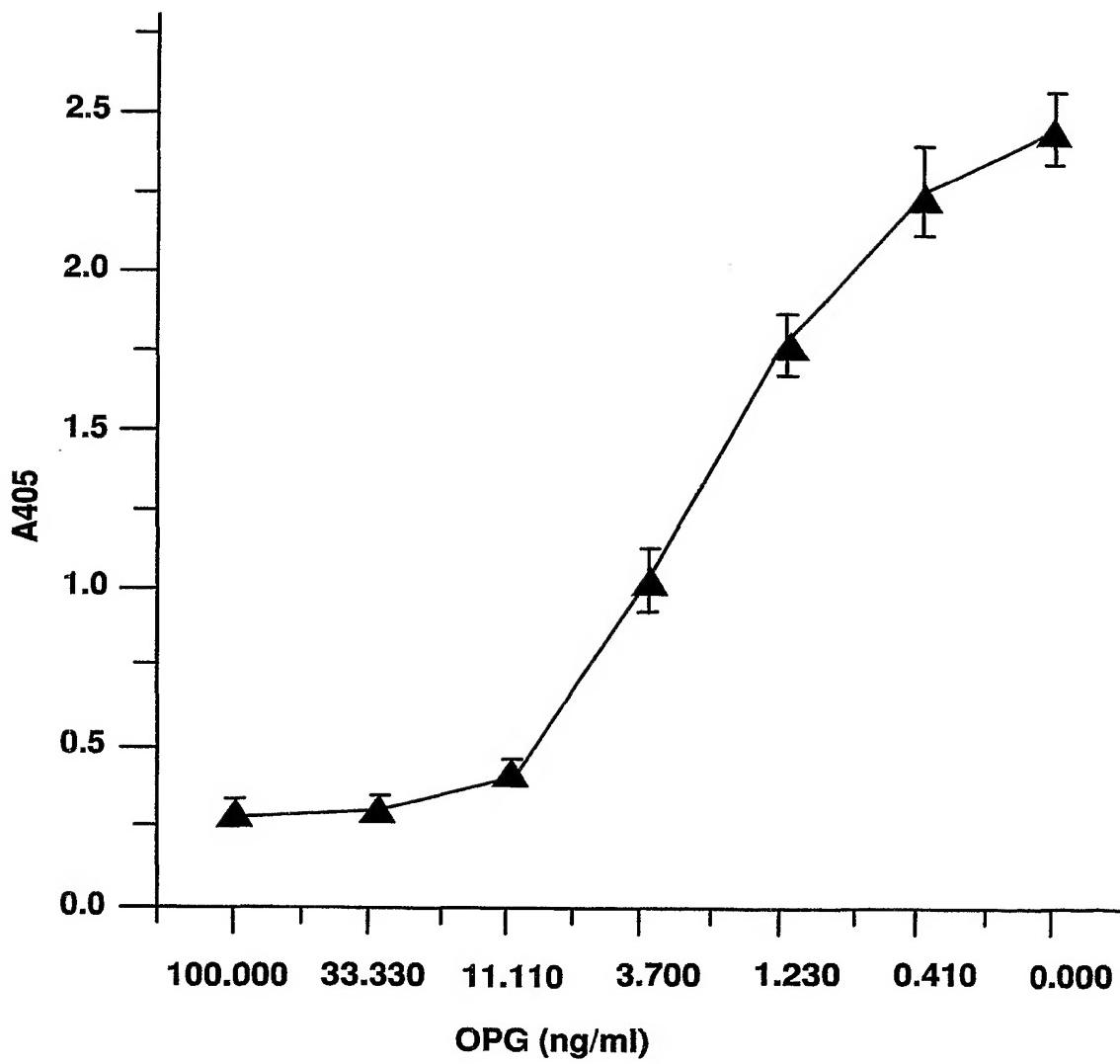
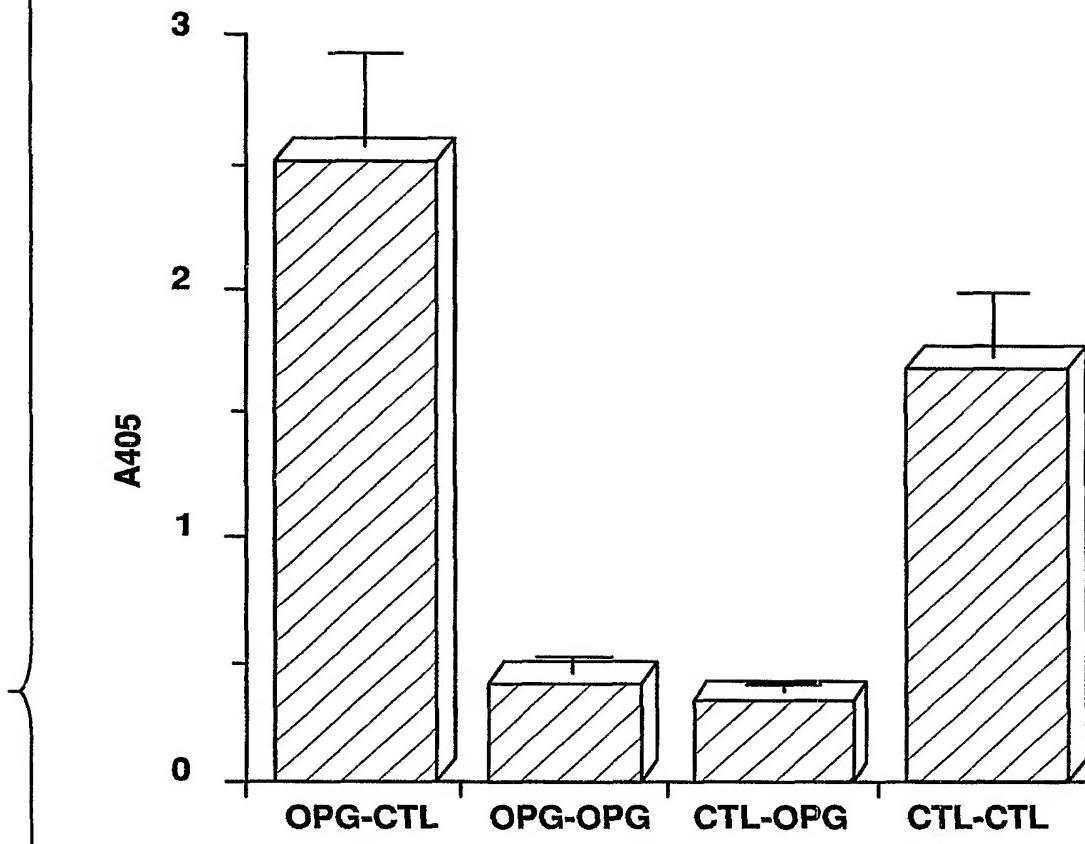
**FIG.20**

FIG.21

**Legend**

**Growth**  
**Bone marrow**  
**cells**  
**CSF -1**

**Intermediate**  
**PGE2 + CSF-1**

**Terminal**  
**ST2 cells**  
**1,25 (OH)2 D3**  
**Dexamethasone**

4 days

2 days

8 - 10 days

**Groups**

CTL - CTL

OPG - CTL

OPG - OPG

OPG - OPG

**OPG**

---

100 ng/ml

---

100 ng/ml

**OPG**

---

---

100 ng/ml

100 ng/ml

FIG.22A

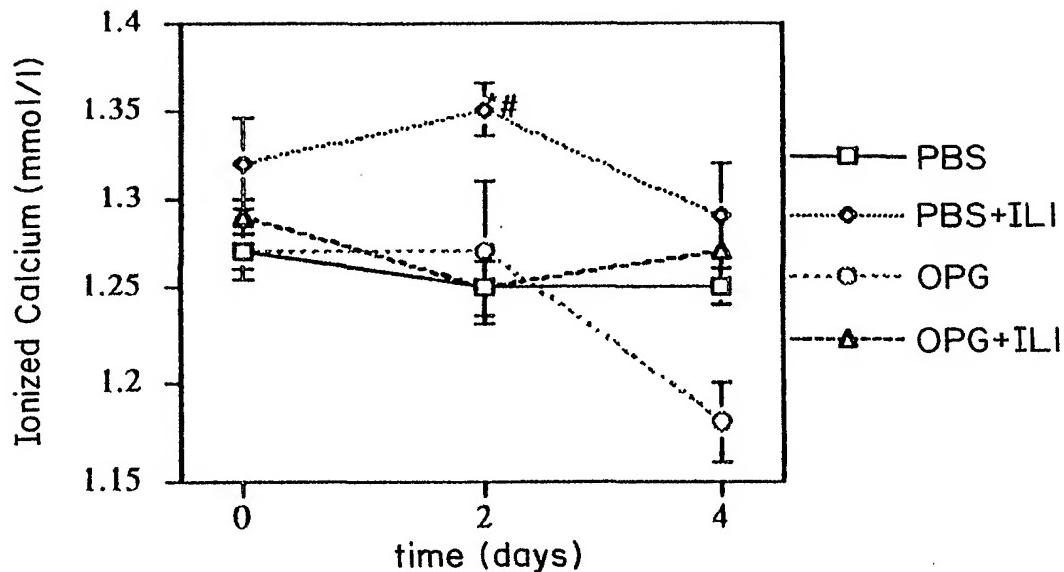
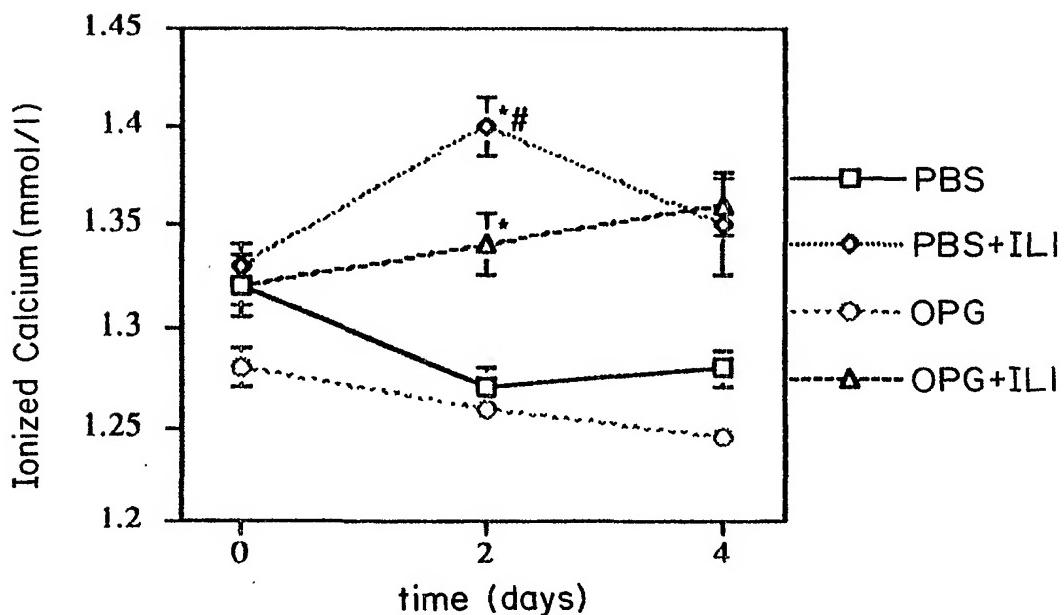


FIG.22B

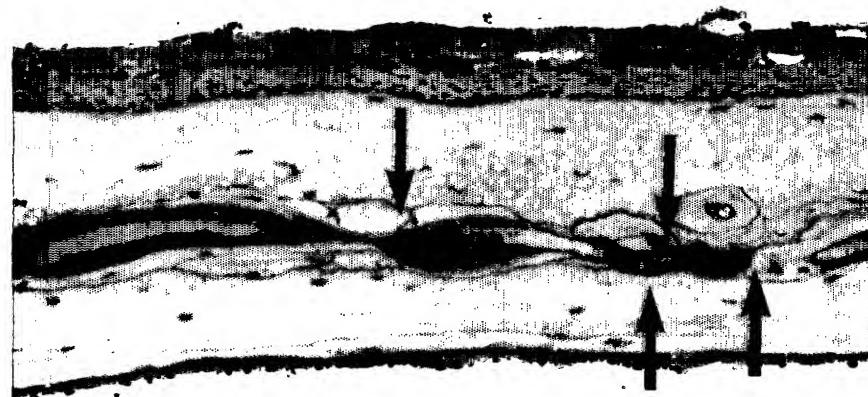


\* Different to PBS,  $p < 0.05$

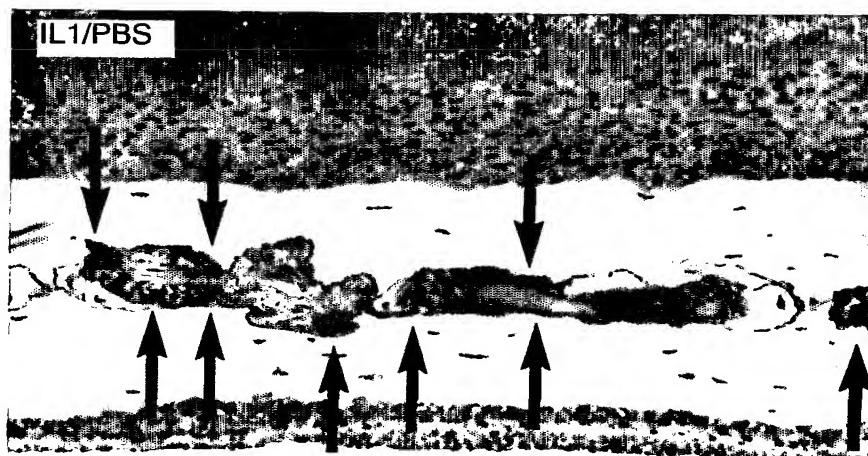
# Different to OPG + IL1,  $p < 0.05$

# FIG.23A

PBS/PBS

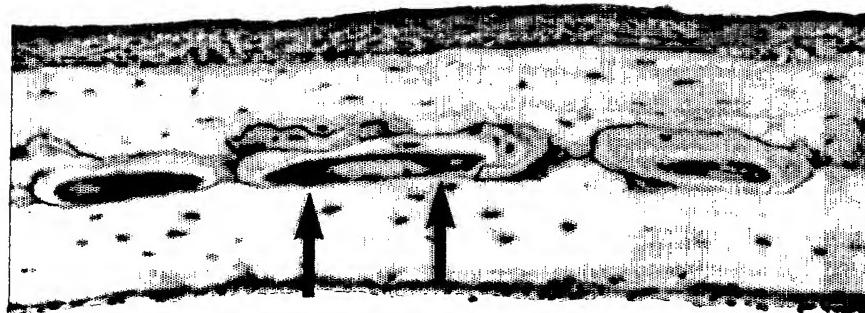


# FIG.23B



# FIG.23C

PBS/OPG



# FIG.23D

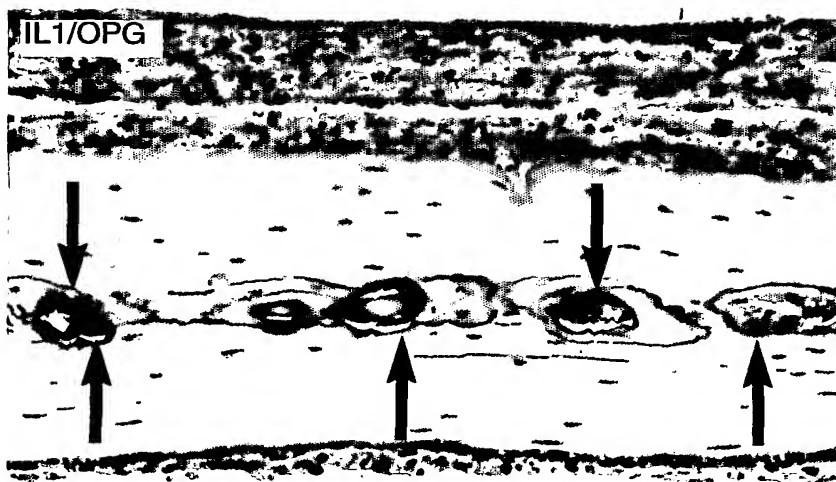


FIG.24B

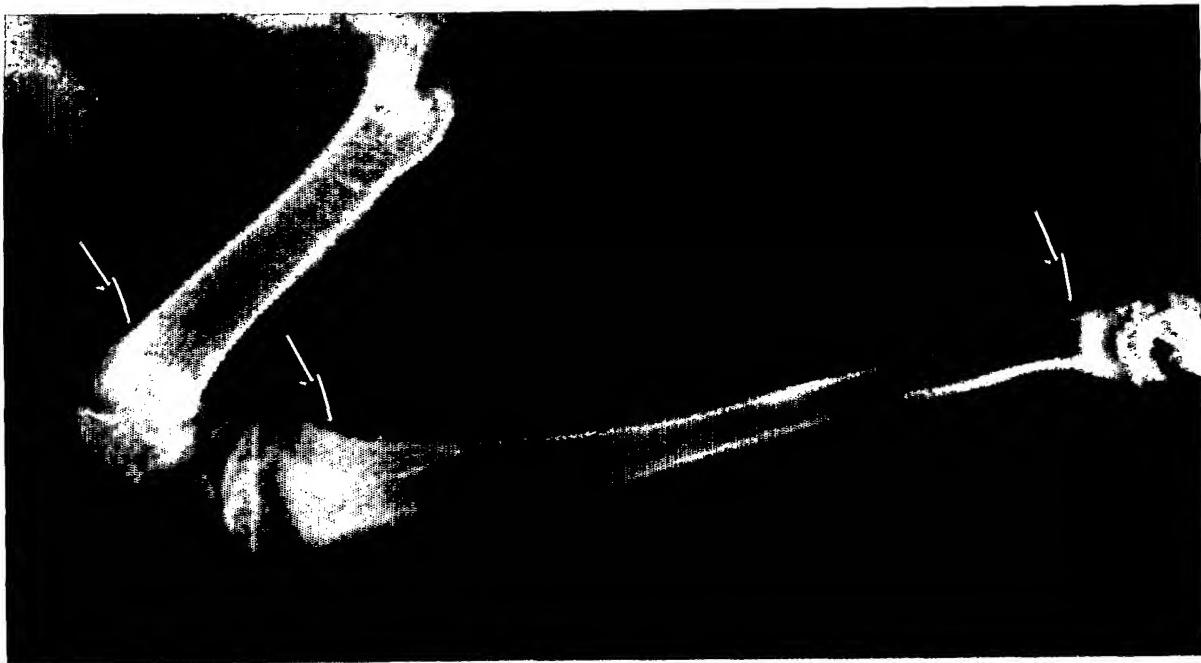


FIG.24A

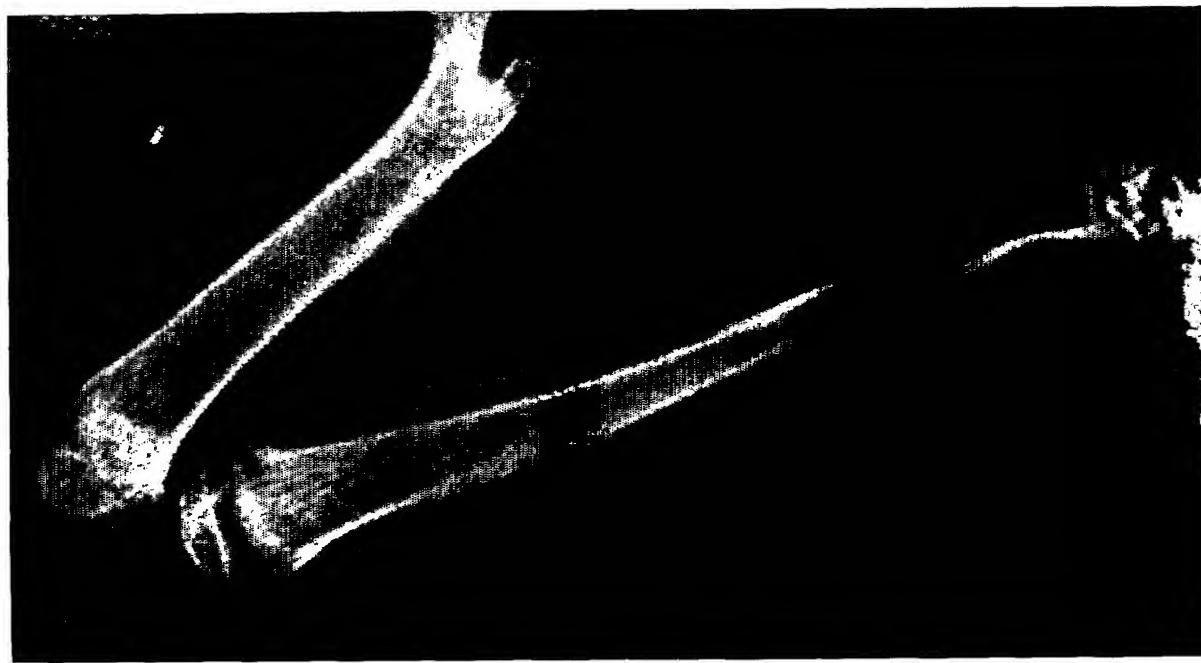
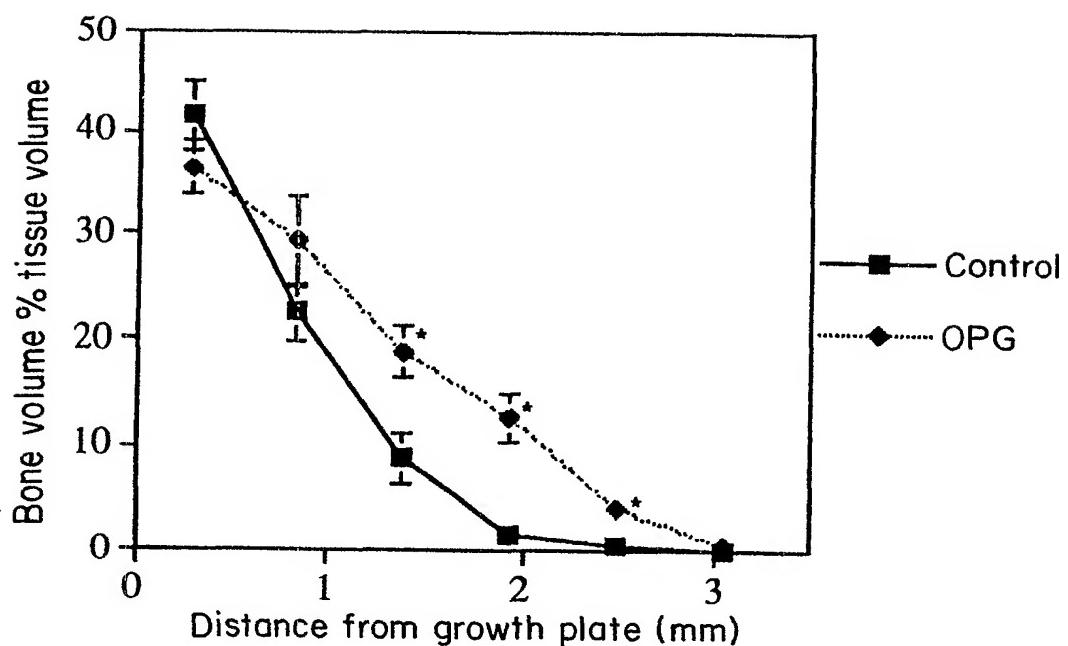


FIG. 25



\* Different to control p < 0.01

**FIG. 26A**



**FIG. 26B**



FIG. 27

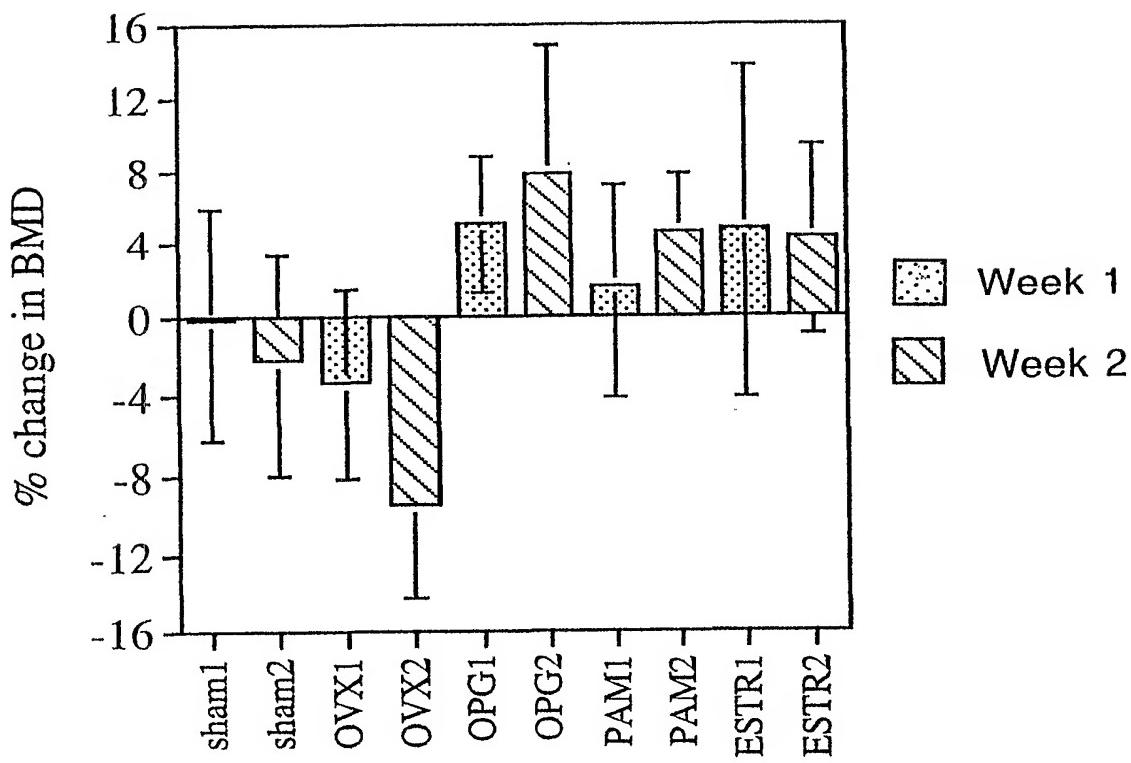
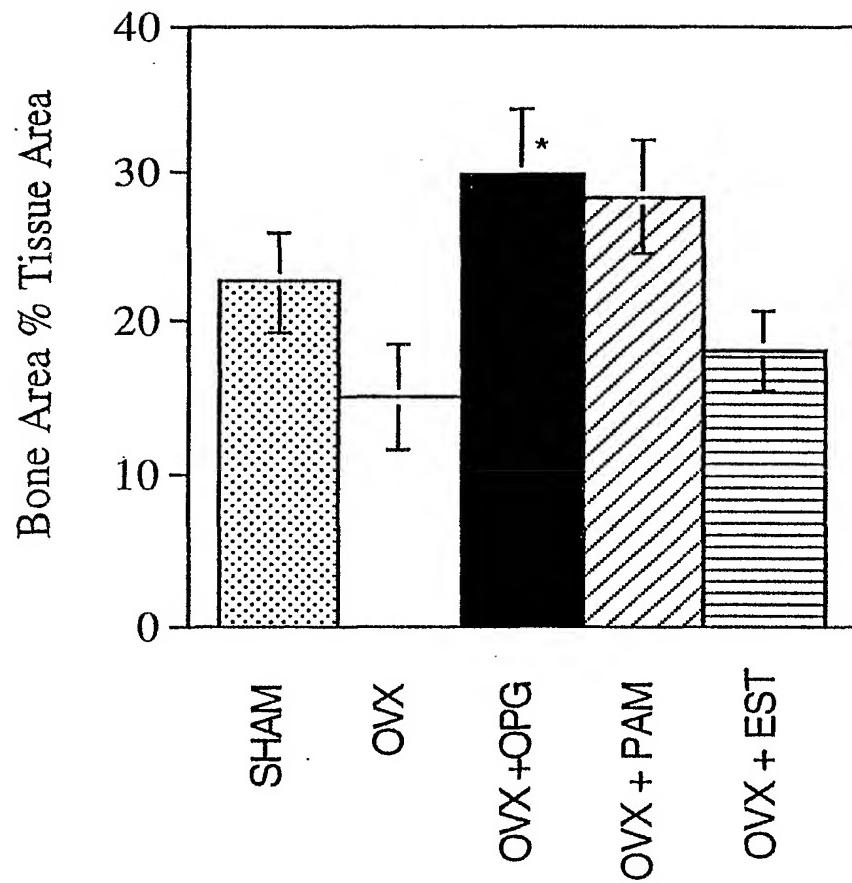


FIG. 28



\* Different to OVX  $p < 0.05$

# FIG. 29A

**DraIII**

```

CATGGGAAATGTCAGAGTGGAGAACCAACACCGAGTGCCACTGCAGCACTTGTATTATCA
1 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 60
GTACCCCTTACAGTCTCACCTCTGGTGTGGCTACGGTGACGTCGTGAACAATAATAGT

CAAATCCTAATAGTTGCAGTGGCCTTGCTGATGATGGCTGACTTGCTAAAAGGAAAA
61 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 120
GTTTAGGATTATCAAACGTCACCCGGAACGACTACTACCGACTGAACGAGTTTCCTTT

TTAATTGTCCAGTGTCTATGGCTTGTGAGATAAAACCCTCCTTTCCTGCCATACCA
121 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 180
AATTAAACAGGTACAGATAACGAAACACTCTATTTGGGAGGAAAAGGAACGGTATGGT

TTTTAACCTGCTTGAGAATATACTGCAGCTTATTGCTTTCCCTTACCTACAATA
181 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 240
AAAAATTGGACGAAACTCTTATATGACGTCGAAATAACGAAAAGAGGAATAGGATGTTAT

TAATCAGTAGTCTGATTTCAAGGAAATGAAATATGGCATTAGCATGACCATAAA
241 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 300
ATTAGTCATCAGAACTAGAAAAGTAAACCTTACTTATACCGTAAATCGTACTGGTATTT

AAGCTGATTCCACTGGAAATAAAAGTCTTTAAATCATCACTCTATCACTGAATTCTAATT
301 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 360
TTCGACTAAGGTGACCTTATTCAGAAAATTAGTAGTAGTGAGATAGTGACTTAAGATTAA

TTTCTGAAAAGTTCAAGCCAGTTACTTTGATAGGATTAACGGAAGGGAGTGAGCCAG
361 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 420
AAAAGACTTTCAAAGTTCGGTCAATGAAAACCTATCCTAATTGCCTCCCTCACTCGGTC

XcmI
|
TGGGTGAGGTGGGTTCCCATGTAGTCAATGGCCTAATACTGGAGAATCTTATTCTAACCA
421 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 480
ACCCACTCCACCCAGGGTACATCAGTTACCGGATTATGACCTCTAGAATAAGATTGGT

AGCCTCCAGAGCAAGCTGTGAGCCCCCTCAGACAGTGGCTACTCATGAGACAGTCCATT
481 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 540
TCGGAAGGTCTCGTCACTCGGGAGTCTGTCACCCGATGAGTACTCTGTCAAGGTAA

GGGGTAAAGGAAGAAAATAACTTCTATTCTATTGCACATTGTCTTAGATGC
541 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 600
CCCCATTCTCTTTATATTGAAGATAAGATAACGTAACAGAAATCTACG

CCATTGGGTGAGTTTATAGAAGTACAGCTACATTAAGGAAACTGATAATAGATA
601 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 660
GGTAAACCCACTCAAATATCTTCACTGTCATGTAATTCTGACTATTATCTAT

```

# FIG. 29B

AGGCTTAAAAAAACTCATTCAATCACCAAGTTGTCAAGATTCCATTCAAAGTGAAGAA  
 661 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 720  
 TCCGAAATTTTGAAAGTAAGTAGTGGTCAAACAGTTCAAGGTAAAGTTCACTTTT

CCAATTCTAACGGGTTGGTAAACACAGCAGATGGCAGGGTAAAAATTAAAGTGAGTGC  
 721 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 780  
 GTTAAAGATTGCCAACCATTTGTGTCGTCTACCGTCCCACTTTTAATTCACTCACG

ATGTACCTTAAAGAAACACTGAAATGCACACACATTACTTAACCTGCTCATTCAATTAT  
 781 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 840  
 TACATGGAAATTCTTGTGACTTTACGTGTGTAATGAATTGGACGAGTAAGTAAATA

TTACATATAGTCTGGGTGTACAAAATTAGAAATAACATATGGGGCGGGGCCTTA  
 841 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 900  
 AATGTATATCAGAACCCACATGTTTAAATCTTATTATGTATACCCCCGCCCCGGAAT

GCTGCACAAATAGGATGCGCGGCGGGCCTGGTAGGGCGGAGCCTAGCTGCACAAATA  
 901 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 960  
 CGACGTGTTATCCTACCGCGCCGGAACCATCCCCGCCTCGGAATCGACGTGTTAT

GGATGCGCGGGCGGGCCTGGTGGGGCGGGGCCTAACGCTGCGCAAGTGGTACACAGCTCA  
 961 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1020  
 CCTACCGCGCCGGAACACCACCCCCGCCCCGATTGACGCGTTACCATGTGTCGAGT

GGGCTGCGATTCGCGCCAAACTTGACGGCAATCCTAGCGTGAAGGCTGGTAGGATTTA  
 1021 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1080  
 CCCGACGCTAAAGCGCGGTTGAAC TGCCGTTAGGATCGCACTCCGACCATCCTAAAAT

TCCCCGCTGCCATCATGGTCGACCATTGAACTGCATCGCGCCGTGTCCAAAATATGG  
 1081 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1140  
 AGGGGCGACGGTAGTACCAAGCTGGTAACTGACGTAGCAGCGGCACAGGGTTTATACC

GGATGGCAAGAACGGAGACCTACCCCTGGCCTCCGCTCAGGAACGAGTTCAAGTACTTCC  
 1141 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1200  
 CCTAACCGTTCTGCCTCTGGATGGGACCGGAGGCAGTCCTGCTCAAGTTCAAGG

AAAGAATGACCACAACCTCTTCAGTGGAAAGTAAACAGAACATCTGGTAGGATGGTAGGA  
 1201 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1260  
 TTTCTTACTGGTGGAGAAGTCACCTCCATTGCTTAGACCAACTAACCCATCCT

AAACCTGGTCTCCATTCTGAGAAGAACATCGACCTTAAAGGACAGAACATATAGTTC  
 1261 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1320  
 TTTGGACCAAGAGGTAAGGACTCTCTTAGCTGGAAATTCTGTCTTAATTATATCAAG

SacI	BstXI

TCAGTAGAGAACTCAAAGAACCAACGAGCTCATTCTGCCAAAGTTGGATG  
 1321 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1380  
 AGTCATCTCTGAGTTCTGGTGGCTCGAGTAAAGAACGGTTCAACCTAC

# FIG. 29C

**AflII**

|

```

ATGCCTTAAGACTTATTGAACAACCGGAATTGGCAAGTAAAGTAGACATGGTTGGATAG
1381 -----+-----+-----+-----+-----+-----+-----+-----+ 1440
TACGGAATTCTGAATAACTGTTGGCCTAACCGTTCATTCATCTGTACCAAACCTATC

TCGGAGGCAGTTCTGTTACCAGGAAGCCATGAATCAACCAGGCCACCTCAGACTCTTG
1441 -----+-----+-----+-----+-----+-----+-----+-----+ 1500
AGCCTCCGTCAAGACAAATGGCCTTCGGTACTTAGTTGGTCCGGTGGAGTCTGAGAAC

TGACAAGGATCATGCAGGAATTGAAAGTGACACGTTTCCCAGAAATTGATTTGGGA
1501 -----+-----+-----+-----+-----+-----+-----+-----+ 1560
ACTGTTCTAGTACGTCTTAAACTTCACTGTGCAAAAGGGCTTTAACTAAACCCCT

AATATAAACTTCTCCCAGAATACCCAGGCGCCTCTCTGAGGTCCAGGAGGAAAAGGCA
1561 -----+-----+-----+-----+-----+-----+-----+-----+ 1620
TTATATTTGAAGAGGGTCTTATGGGTCCGCAGGAGAGACTCCAGGTCCCTCTTTCCGT

TCAAGTATAAGTTGAAGTCTACGAGAAGAAAGACTAACAGGAAGATGCTTCAAGTTCT
1621 -----+-----+-----+-----+-----+-----+-----+-----+ 1680
AGTTCATATTCAAACCTCAGATGCTCTTCTGATTGTCTTACGAAAGTTCAAGA

```

**BglII**

|

```

CTGCTCCCCTCTAAAGCTATGCATTTATAAGACCATGGACTTTGCTGGCTTTAGA
1681 -----+-----+-----+-----+-----+-----+-----+-----+ 1740
GACGAGGGGAGGATTCGATACGTAaaaATTCTGGTACCTGAAACGACCGAAATCT

TCTGAAACACTGAAATTGCTGCTCTCATCTCAGTGAGATTCCAAGGATAGTACAGT
1741 -----+-----+-----+-----+-----+-----+-----+-----+ 1800
AGACTTTGTGACTTTAACAGACGAAGAGTAGAAGTCACTCTAAGGTTCTATCATGTCA

GACAGAACAAAGAATAGGCACTCTCTACAAAAAAAAGAAAGAAAAACTAAGTAATAGCAA
1801 -----+-----+-----+-----+-----+-----+-----+-----+ 1860
CTGTCTTGTCTTATCCGTGAGAGATGTTTTCTTTGATTCTTACCGTT

GCATAATAGCTACTGTTAAGAACTCAGAGATAATGAATTGAGAATGGACTTGCTGAAA
1861 -----+-----+-----+-----+-----+-----+-----+-----+ 1920
CGTATTATCGATGACAATTCTGAGTCTCTATTACTTAACCTTACCTATGACGAACCTT

TGAAAATTAAAGTTAGAAACTAAACTTATAAAAATAAAAATGAGCATTAAAAAA
1921 -----+-----+-----+-----+-----+-----+-----+-----+ 1980
ACTTTAAATTATTCAATCTTGATTGAAATATTTTATTTTACTCGTAATTTTT

```

**NheI**

|

```

AAAAAAAAAAAAAAAAACCCCCCCCCCCCCCTGCAGCCAAGCTAGCTTGGAAATC
1981 -----+-----+-----+-----+-----+-----+-----+-----+ 2040
TTTTTTTTTTTTTTGGGGGGGGGGGGGACGTCGGTTCGATCGAACCTTAG

```

## FIG. 29D

BspLU11I

|  
 AGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAA  
 2041 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2100  
 TCCCCTATTGCGTCCTTCTTGTACACTCGTTCCGGTCGTTCCGGTCCTGGCATT  
  
 AAAGGCCGCGTTGCTGGCGTTTCCATAGGCTCCGCCCTGACGAGCATCACAAAAA  
 2101 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2160  
 TTTCCGGCGAACGACCGAAAAAGGTATCCGAGGCGGGGACTGCTCGTAGTGT  
  
 TCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATAACCAGGC  
 2161 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2220  
 AGCTGCGAGTTCACTCCACCGCTTGGGCTGTCCTGATATTCTATGGTCCGAAAGG  
  
 CCCTGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCCCTGCCGTTACCGGATACCTGTC  
 2221 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2280  
 GGGACCTTCGAGGGAGCACCGAGAGGACAAGGCTGGGACGGGAATGGCCTATGGACAG  
  
 CGCCTTCCTCCCTCGGGAAAGCGTGGCGCTTCTCATAGCTCACGCTGTAGGTATCTCAG  
 2281 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2340  
 GCGGAAAGAGGGAAAGCCCTCGCACCGCGAAAGAGTATCGAGTGCACATCCATAGAGTC  
  
 TTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGCACGAACCCCCCGTTAGCCCGA  
 2341 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2400  
 AAGCCACATCCAGCAAGCGAGGTTCGACCCGACACACGTGCTGGGGCAAGTCGGGCT  
  
 CCGCTGCGCCTATCCGTAACACTCGTCTTGAGTCCAACCCGTAAGACACGACTTATC  
 2401 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2460  
 GGCGACCGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGCCATTCTGCTGAATAG  
  
 GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC  
 2461 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2520  
 CGGTGACCGTCGTCGGTGACCATTGTCCTAATCGTCTCGCTCCATACATCCGCCACGATG  
  
 AGAGTTCTTGAAGTGGTGGCCTAACACTACGGCTACACTAGAAGGACAGTATTTGGTATCTG  
 2521 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2580  
 TCTCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTCTGTCAATAACCAGATAG  
  
 CGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTGATCCGGAAACA  
 2581 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2640  
 GCGAGACGACTTCGGTCAATGGAAGCCTTTCTCAACCATCGAGAACTAGGCCGTTGT

HgiEII

|  
 AACCAACCGCTGGTAGCGGTGGTTTTGTTGCAAGCAGCAGATTACGCGCAGAAAAAA  
 2641 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2700  
 TTGGTGGCGACCATGCCACCAAAAAACAAACGTTCGTCGTCTAATGCGCGTCTTTT  
  
 AGGATCTCAAGAAGATCCTTGATCTTCTACGGGTCTGACGCTCAGTGGAACGAAAA  
 2701 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2760  
 TCCTAGAGTTCTCTAGGAAACTAGAAAAGATGCCAGACTGCGAGTCACCTGCTTT

# FIG. 29E

CTCACGTTAAGGGATTTGGTCATGAGATTATCAAAAGGATCTCACCTAGATCCTTT  
 2761 -----+-----+-----+-----+-----+-----+-----+-----+ 2820  
 GAGTGCATTCCCTAAAACCACTACTCTAATAGTTTCCTAGAAGTGGATCTAGGAAAA  
 AAATTAATAATGAAGTTTAAATCAATCTAAAGTATATATGAGTAAACTGGTCTGACAG  
 2821 -----+-----+-----+-----+-----+-----+-----+-----+ 2880  
 TTTAATTTTACTTCAAATTTAGTTAGATTCATATATACTCATTTGAACCAGACTGTC  
 TTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTCGTTATCCAT  
 2881 -----+-----+-----+-----+-----+-----+-----+-----+ 2940  
 AATGGTTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAAAGCAAGTAGGTA  
 AGTTGCCTGACTCCCCGTGTTAGATAACTACGATAACGGGAGGGCTTACCATCTGGCCC  
 2941 -----+-----+-----+-----+-----+-----+-----+-----+ 3000  
 TCAACGGACTGAGGGCAGCACATCTATTGATGCTATGCCCTCCGAATGGTAGACCGGG  
 CAGTGCTGCAATGATAACCGCGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAA  
 3001 -----+-----+-----+-----+-----+-----+-----+-----+ 3060  
 GTCACGACGTTACTATGGCGCTCTGGTGCGAGTGGCCGAGGTCTAAATAGTCGTTATTT  
 CCAGCCAGCCGAAGGGCGAGCGCAGAAGTGGTCTGCAACTTATCCGCCTCCATCCA  
 3061 -----+-----+-----+-----+-----+-----+-----+-----+ 3120  
 GGTCGGTCGGCCTTCCCGCTCGCGTCTCACCGAGCGTTGAAATAGCGGGAGGTAGGT  
 GTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTCGCCAGTTAATAGTTGCGCAA  
 3121 -----+-----+-----+-----+-----+-----+-----+-----+ 3180  
 CAGATAATTAACAACGGCCCTCGATCTCATTCAAGCGGTCAATTATCAAACCGCGTT  
 CGTTGTTGCCATTGCTGCAGGCATCGTGGTCACGCTCGTCGTTGGTATGGCTTCATT  
 3181 -----+-----+-----+-----+-----+-----+-----+-----+ 3240  
 GCAACAACGGTAACGACGTCCGTAGCACCAAGTGCAGCAGCAAACCATACCGAAGTAA  
 CAGCTCCGGTTCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGC  
 3241 -----+-----+-----+-----+-----+-----+-----+-----+ 3300  
 GTCGAGGCCAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGGGTACAACACGTTTTCG  
 EaeI  
 PvuI            GdII  
 |                |  
 GGTTAGCTCCTCGGTCTCCGATCGTGTAGAAGTAAGTTGGCCGCAGTGTATTCCT  
 3301 -----+-----+-----+-----+-----+-----+-----+-----+ 3360  
 CCAATCGAGGAAGCCAGGAGGCTAGCAACAGTCTCATTCAACCGCGTCACAATAGTGA  
 CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC  
 3361 -----+-----+-----+-----+-----+-----+-----+-----+ 3420  
 GTACCAATACCGTCGTGACGTATTAAGAGAATGACAGTACGGTAGGCATTCTACGAAAAG  
 BcgI  
 |  
 TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGGCGACCGAGTTG  
 3421 -----+-----+-----+-----+-----+-----+-----+-----+ 3480  
 ACACGTGACCAACTCATGAGTTGGTCAGTAAGACTCTTATCACATACGCCGCTGGCTAAC

**FIG. 29F**

CTCTTCCCCGGCGTCAACACGGGATAATACCGGCCACATAGCAGAACTTTAAAAGTGCT  
 3481 -----+-----+-----+-----+-----+-----+-----+-----+ 3540  
 GAGAACGGGCCGCAGTTGTGCCCTATTATGGCGCGGTGTACGTCTTGAAATTTCACGA  
  
 CATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATC  
 3541 -----+-----+-----+-----+-----+-----+-----+-----+ 3600  
 GTAGTAACCTTTGCAAGAAGCCCCGCTTGAGAGTTCTAGAATGGCGACAACACTAG  
  
 CAGTTCGATGTAACCCACTCGTGCACCCAACGTGATCTTCAGCATCTTACTTTACCCAG  
 3601 -----+-----+-----+-----+-----+-----+-----+-----+ 3660  
 GTCAAGCTACATTGGGTGAGCACGTGGGTTGACTAGAAGTCGTAGAAAATGAAAGTGGTC  
  
 CGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGAAAAAGGGATAAGGGCGAC  
 3661 -----+-----+-----+-----+-----+-----+-----+-----+ 3720  
 GCAAAGACCCACTCGTTTGTCCCTTCGTTTACGGCGTTTCCCTTATTCCCGCTG  
  
 SspI  
 |  
 ACGGAAATGTTGAATACTCATACTCTTCCTTTCAATATTATTGAAGCATTATCAGGG  
 3721 -----+-----+-----+-----+-----+-----+-----+-----+ 3780  
 TGCCTTACAACTTATGAGTATGAGAAGGAAAAGTTATAAACTTCGTAAATAGTCCC  
  
 TTATTGTCTCATGAGCGGATAACATATTGAATGTATTAGAAAAATAACAAATAGGGT  
 3781 -----+-----+-----+-----+-----+-----+-----+-----+ 3840  
 AATAACAGAGTACTCGCCTATGTATAAACTTACATAAAATCTTTATTGTTATCCCCA  
  
 TCCGCGCACATTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGAC  
 3841 -----+-----+-----+-----+-----+-----+-----+-----+ 3900  
 AGGCGCGTGTAAAGGGCTTTCACGGTGGACTGCAGATTCTTGGTAATAATAGTACTG  
  
 ATTAACCTATAAAAATAGCGTATCACGAGGCCCTTCGTCTTCAAGAATTCCCTGTGGA  
 3901 -----+-----+-----+-----+-----+-----+-----+-----+ 3960  
 TAATTGGATATTTTATCCGATAGTGCCTCCGGAAAGCAGAAGTTCTTAAGGGACACCT  
  
 ATGTGTGTCAAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAA  
 3961 -----+-----+-----+-----+-----+-----+-----+-----+ 4020  
 TACACACAGTCATCCCACACCTTCAGGGTCCGAGGGTCCGTCCGTCTTCATACGTT  
  
 GCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCA  
 4021 -----+-----+-----+-----+-----+-----+-----+-----+ 4080  
 CGTACGTAGAGTTAATCAGTCGTTGGTCCACACCTTCAGGGTCCGAGGGTCCGTCCGT  
  
 GAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCT  
 4081 -----+-----+-----+-----+-----+-----+-----+-----+ 4140  
 CTTCATACGTTCGTAGAGTTAATCAGTCGTTGGTATCAGGGCGGGATTGAGGCG  
  
 CCATCCCCCTAATTCCGCCAGTTCCGCCATTCTCCGCCATGGCTGACTAATT  
 4141 -----+-----+-----+-----+-----+-----+-----+-----+ 4200  
 GGTAGGGCGGGGATTGAGGCGGGTCAAGGCGGGTAAGAGGGGGTACCGACTGATTAAA

## FIG. 29G

## SfiI

TTTTTATTATGCAGAGGCCGAGGCCGCCTCGGCCTTGAGCTATTCCAGAAGTAGTGAG  
 4201 -----+-----+-----+-----+-----+-----+ 4260  
 AAAAAATAAAATACGTCTCCGGCTCCGGAGGCCGGAGACTCGATAAGGTCTTCATCACTC

## AvrII

GAGGCTTTTGGAGGCCTAGGCTTGCAAAAAGCTGGTCGAGGCTCGCATCTCTCCTT  
 4261 -----+-----+-----+-----+-----+-----+ 4320  
 CTCCGAAAAAACCTCCGGATCCGAAACGTTTCGACCAGCTCCGAGCGTAGAGAGGAA  
  
 CACCGCCCCGCCCTACCTGAGGCCGCATCCACGCCGGTTGAGTCGCCTGCCGC  
 4321 -----+-----+-----+-----+-----+-----+ 4380  
 GTGCGCGGGCGGCGGGATGGACTCCGGCGTAGGTGCGGCCAACTCAGCGCAAGACGGCG  
  
 CTCCCGCTGTGGTGCCTCCTGAACTGCGTCCGCCGTCTAGGTAAGTTAAAGCTCAGGT  
 4381 -----+-----+-----+-----+-----+-----+ 4440  
 GAGGGCGGACACCAACGGAGGACTTGACGCAGGCCAGATCCATTCAAATTGAGTCCA

## NgoAIV

CGAGACCGGGCCTTGTCCGGCGTCCCTGGAGCCTACCTAGACTCAGCCGGCTCTCCA  
 4441 -----+-----+-----+-----+-----+-----+ 4500  
 GCTCTGGCCCGGAAACAGGCCGCAGGGAACCTCGGATGGATCTGAGTCGGCCGAGAGGT  
  
 CGCTTGCCTGACCCTGCTTGTCAACTCTACGTCTTGTTCGTTCTGTTCTGCGCC  
 4501 -----+-----+-----+-----+-----+-----+ 4560  
 GCGAACCGGACTGGGACGAACGAGTTGAGATGCAGAAACAAAGCAAAGACAAGACCGG

## HpaI

GTTACAGATCCGTCGAGGAACGTAAAAACAGAAAGTTAACTGGTAAGTTAGTCTTTT  
 4561 -----+-----+-----+-----+-----+-----+ 4620  
 CAATGTCTAGGCAGCTCCTTGACTTTGGCTTCAATTGACCATTCAAATCAGAAAAA

## Psp5II BamHI

GTCTTTTATTTCAGGTCCGGATCCGGTGGTGGTGCAAATCAAAGAACTGCTCCTCAGTG  
 4621 -----+-----+-----+-----+-----+-----+ 4680  
 CAGAAAATAAAGTCCAGGGCCTAGGCCACCACGTTAGTTCTGACGAGGAGTCAC  
  
 GATGTTGCCTTACTTCTAGGCCTGTACGGAAGTGTACTTCTGCTCTAAAGCTGCTGC  
 4681 -----+-----+-----+-----+-----+-----+ 4740  
 CTACAACGGAAATGAAGATCCGGACATGCCCTCACAAATGAAGACGAGATTTGACGACG

## HindIII XbaI

## BssHII

AACAAGCTTCTAGACCACCATGAACAAGTTGCTGTGCTGCGCGCTCGTGTGACAT  
 4741 -----+-----+-----+-----+-----+-----+ 4800  
 TTGTTCGAAGATCTGGTGGTACTTGTCAACGACACGACGCGAGCACAAAGACCTGTA  
 b M N K L L C C A L V F L D I -

## FIG. 29H

CTCCATTAAGTGGACCACCCAGGAAACGTTCCCTCAAAGTACCTCATTATGACGAAGA  
 4801 -----+-----+-----+-----+-----+-----+ 4860  
 GAGGTAATTCACCTGGTGGGTCTTGCAAAAGGAGGTTCATGGAAGTAATACTGCTTCT  
 b S I K W T T Q E T F P P K Y L H Y D E E -

KpnI

AACCTCTCATCAGCTGTTGTGACAAATGTCCTCTGGTACCTACCTAAAACAACACTG  
 4861 -----+-----+-----+-----+-----+-----+ 4920  
 TTGGAGAGTAGTCGACAACACACTGTTACAGGAGGACATGGATGGATTTGTTGTGAC  
 b T S H Q L L C D K C P P G T Y L K Q H C -

TACAGCAAAGTGGAAAGACCGTGTGCGCCCCCTGCCCTGACCACTACTACACAGACAGCTG  
 4921 -----+-----+-----+-----+-----+-----+ 4980  
 ATGTCGTTTACACCTCTGGCACACGCGGGAACGGACTGGTATGATGTGTCTGTCGAC  
 b T A K W K T V C A P C P D H Y Y T D S W -

GCACACCAAGTGACGAGTGTCTATACTGCAGCCCCGTGTGCAAGGAGCTGCAGTACGTCAA  
 4981 -----+-----+-----+-----+-----+-----+ 5040  
 CGTGTGGTCACTGTCACAGATATGACGTCGGGCACACGTTCCCTGACGTCATGCAGTT  
 b H T S D E C L Y C S P V C K E L Q Y V K -

RleAI BsmI

GCAGGAGTGCAATCGCACCCACAACCGCGTGTGCAATGCAAGGAAGGGCGTACCTTGA  
 5041 -----+-----+-----+-----+-----+-----+ 5100  
 CGTCCTCACGTTAGCGTGGGTGTTGGCGCACACGCTTACGTTCCCTCCCGCATGGAAC  
 b Q E C N R T H N R V C E C K E G R Y L E -

GATAGAGTTCTGCTTGAACATAGGAGCTGCCCTCTGGATTGGAGTGGTGCAGCTGG  
 5101 -----+-----+-----+-----+-----+-----+ 5160  
 CTATCTCAAGACGAACCTTGATCCTCGACGGGAGGACCTAACCTCACACGTTGACC  
 b I E F C L K H R S C P P G F G V V Q A G -

BsmBI

AACCCCAGAGCGAAATACAGTTGCAAAAGATGTCCAGATGGGTTCTCTCAAATGAGAC  
 5161 -----+-----+-----+-----+-----+-----+ 5220  
 TTGGGGTCTCGCTTATGTCAAACGTTTCTACAGGTCTACCCAAGAAGAGTTACTCTG  
 b T P E R N T V C K R C P D G F F S N E T -

GTCATCTAAAGCACCTGTAGAAAACACACAAATTGCAGTGTCTTGGTCTCTGCTAAC  
 5221 -----+-----+-----+-----+-----+-----+ 5280  
 CAGTAGATTCGTGGACATCTTGTGTGTTAACGTCACAGAAACCAAGAGGACGATTG  
 b S S K A P C R K H T N C S V F G L L L T -

BspEI

TCAGAAAGGAAATGCAACACACGACAACATATGTTCCGGAAACAGTGAATCAACTCAAA  
 5281 -----+-----+-----+-----+-----+-----+ 5340  
 AGTCTTTCTTACGTTGTGCTGTTGTATACAAGGCCTTGTCACTTAGTTGAGTTT  
 b Q K G N A T H D N I C S G N S E S T Q K -

# FIG. 29 I

**SalI**  
 |  
 AGTCGACAAA ACTCACACATGCCACC GTGCCAGCACCTGA ACTCCTGGGGGACCGTC  
 5341 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 5400  
 TCAGCTTTGAGTGTGTACGGTGGCACGGTCTGGACTTGAGGACCCCCCTGGCAG  
 b V D K T H T C P P C P A P E L L G G P S -

AGTCTCCTCTTCCCCCAAAACCCAAGGACACCCCATGATCTCCGGACCCCTGAGGT  
 5401 -----+-----+-----+-----+-----+-----+-----+-----+ 5460  
 TCAGAAGGAGAAGGGGGTTTGGGTTCTGTGGAGTACTAGAGGGCCTGGGACTCCA  
 b V F L F P P K P K D T L M I S R T P E V -

**BtrI**  
 |  
 CACATGCGTGGTGGTGACGTGAGCCACGAAGACCTGAGGTCAAGTTCAACTGGTACGT  
 5461 -----+-----+-----+-----+-----+-----+-----+-----+ 5520  
 GTGTACGCACCACCCACTGCACTCGGTCTGGACTCCAGTTCAAGTTGACCATGCA  
 b T C V V V D V S H E D P E V K F N W Y V -

**SacII**  
 |  
 GGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGGGGAGGAGCAGTACAACACAC  
 5521 -----+-----+-----+-----+-----+-----+-----+-----+ 5580  
 CCTGCCGCACCTCCACGTATTACGGTTCTGGCGCCCTCGTCATGTTGCTG  
 b D G V E V H N A K T K P R E E Q Y N S T -

GTACCGTGTGGTCAGCGTCCTCACCGTCTGCACCAGGACTGGCTGAATGGCAAGGAGTA  
 5581 -----+-----+-----+-----+-----+-----+-----+-----+ 5640  
 CATGGCACACCAGTCGCAGGAGTGGCAGGACGTGGCTCTGACCGACTTACCGTTCTCAT  
 b Y R V V S V L T V L H Q D W L N G K E Y -

CAAGTGCAAGGTCTCAAACAAAGCCCTCCCAGCCCCATCGAGAAAACCATCTCAAAGC  
 5641 -----+-----+-----+-----+-----+-----+-----+-----+ 5700  
 GTTCACGTTCCAGAGGTTGTTGGGAGGGTAGCTCTTTGGTAGAGGTTTCG  
 b K C K V S N K A L P A P I E K T I S K A -

**SmaI**  
 |  
 CAAAGGGCAGCCCCGAGAACCAACAGGTGTACACCCATGCCGGATGAGCTGAC  
 5701 -----+-----+-----+-----+-----+-----+-----+-----+ 5760  
 GTTCCCGTCGGGCTTGGTCCACATGTGGACGGGGTAGGGCCCTACTCGACTG  
 b K G Q P R E P Q V Y T L P P S R D E L T -

CAAGAACCGAGTCAGCCTGACCTGCCTGGTCAAAGGCTCTATCCCAGCGACATGCCGT  
 5761 -----+-----+-----+-----+-----+-----+-----+-----+ 5820  
 GTTCTGGTCCAGTCGGACTGGACGGACCAGTTCCGAAGATAGGGTCGCTGTAGCGGCA  
 b K N Q V S L T C L V K G F Y P S D I A V -

GGAGTGGGAGAGCAATGGCAGCCGGAGAACAACTACAAGACCACGCCCTCCGTGCTGGA  
 5821 -----+-----+-----+-----+-----+-----+-----+-----+ 5880  
 CCTCACCCCTCTCGTTACCCGTGGCCTCTGTTGATGTTCTGGTGGAGGGCACGACCT  
 b E W E S N G Q P E N N Y K T T P P V L D -

# FIG. 29J

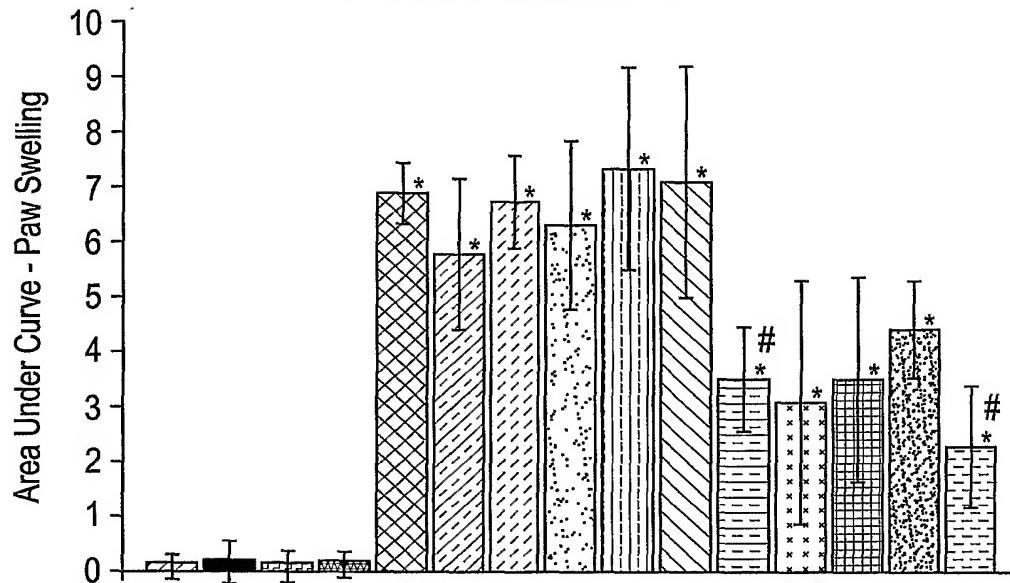
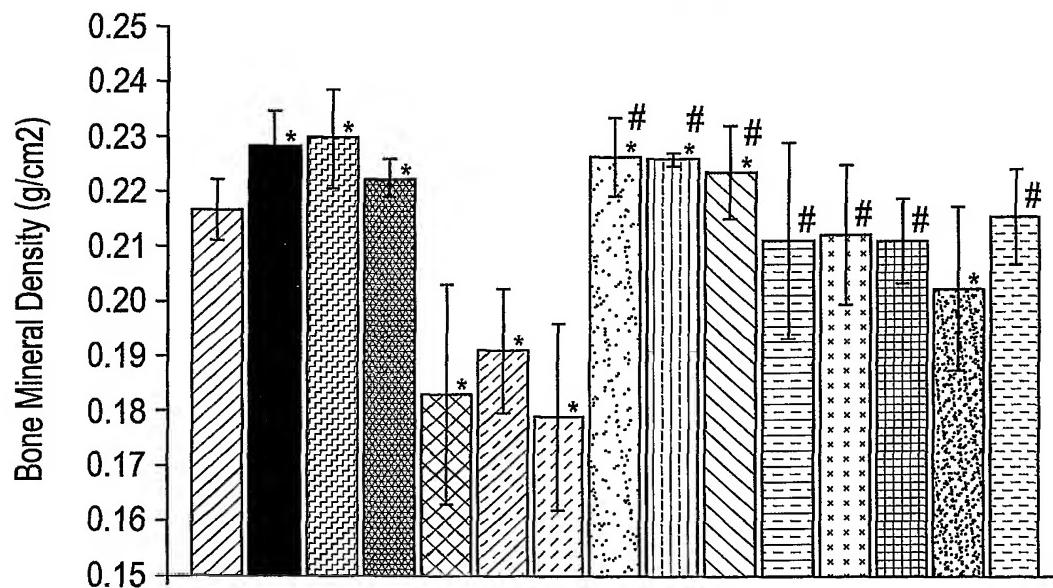
AarI

CTCCGACGGCTCCTCTTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCA  
5881 -----+-----+-----+-----+-----+-----+-----+-----+ 5940  
GAGGCTGCCGAGGAAGAAGGAGATGTCGTTGAGTGGCACCTGTTCTCGTCCACCGTCGT  
b S D G S F F L Y S K L T V D K S R W Q Q -

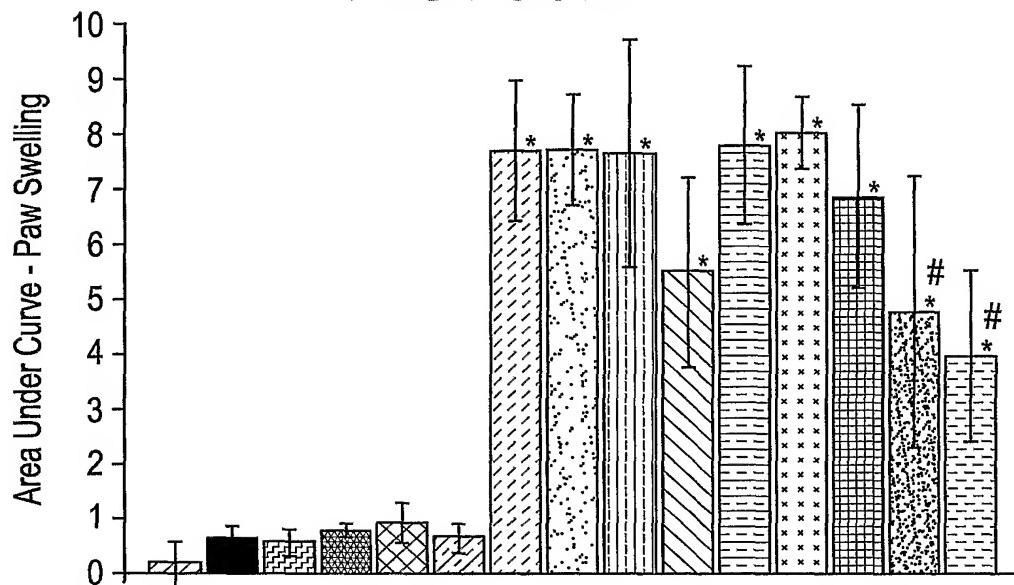
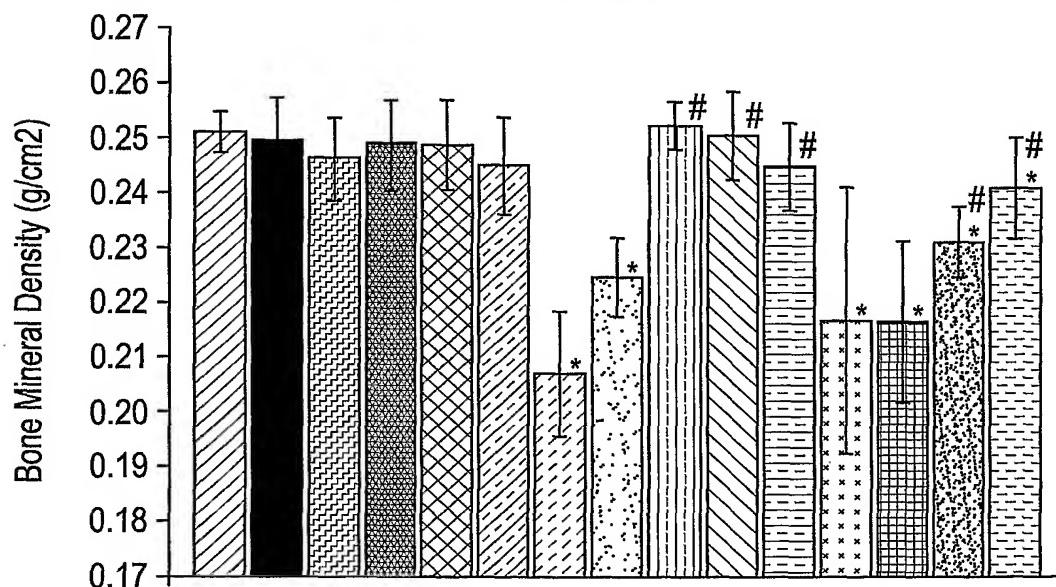
SapI

GGGAAACGTCTTCTCATGCTCCGTATGCATGAGGCTCTGCACAACCACTACACGCAGAA  
5941 -----+-----+-----+-----+-----+-----+-----+-----+ 6000  
CCCCCTGCAGAACAGACTACGAGGCACTACGTACTCCGAGACGTGTTGGTATGTGCGTCTT  
b G N V F S C S V M H E A L H N H Y T Q K -

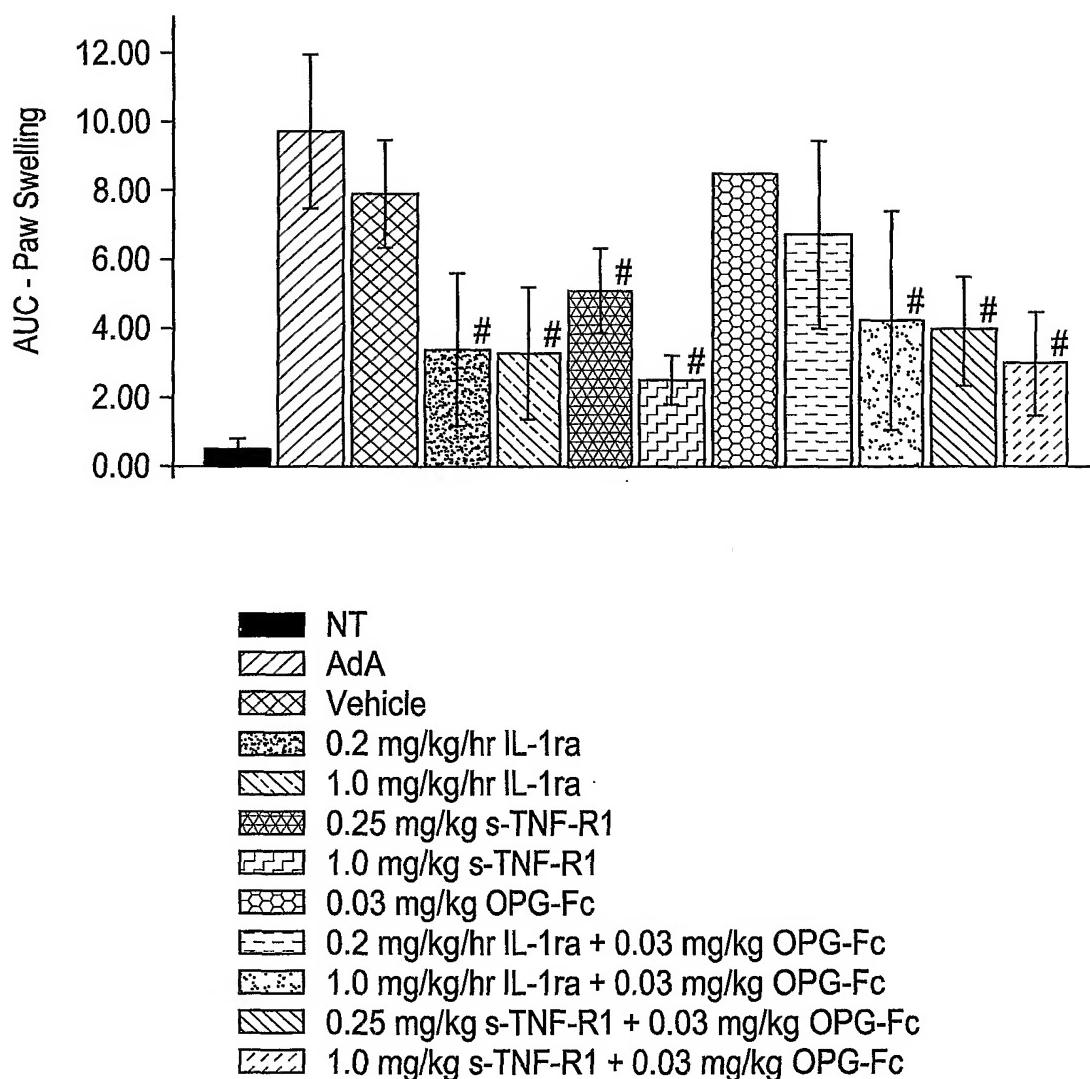
GAGCCTCTCCCTGTCTCCGGTAAATGATAACTCGAC  
6001 -----+-----+-----+-----+-----+ 6037  
CTCGGAGAGGGACAGAGGCCATTACTATTGAGCTG  
b S L S L S P G K \* \*

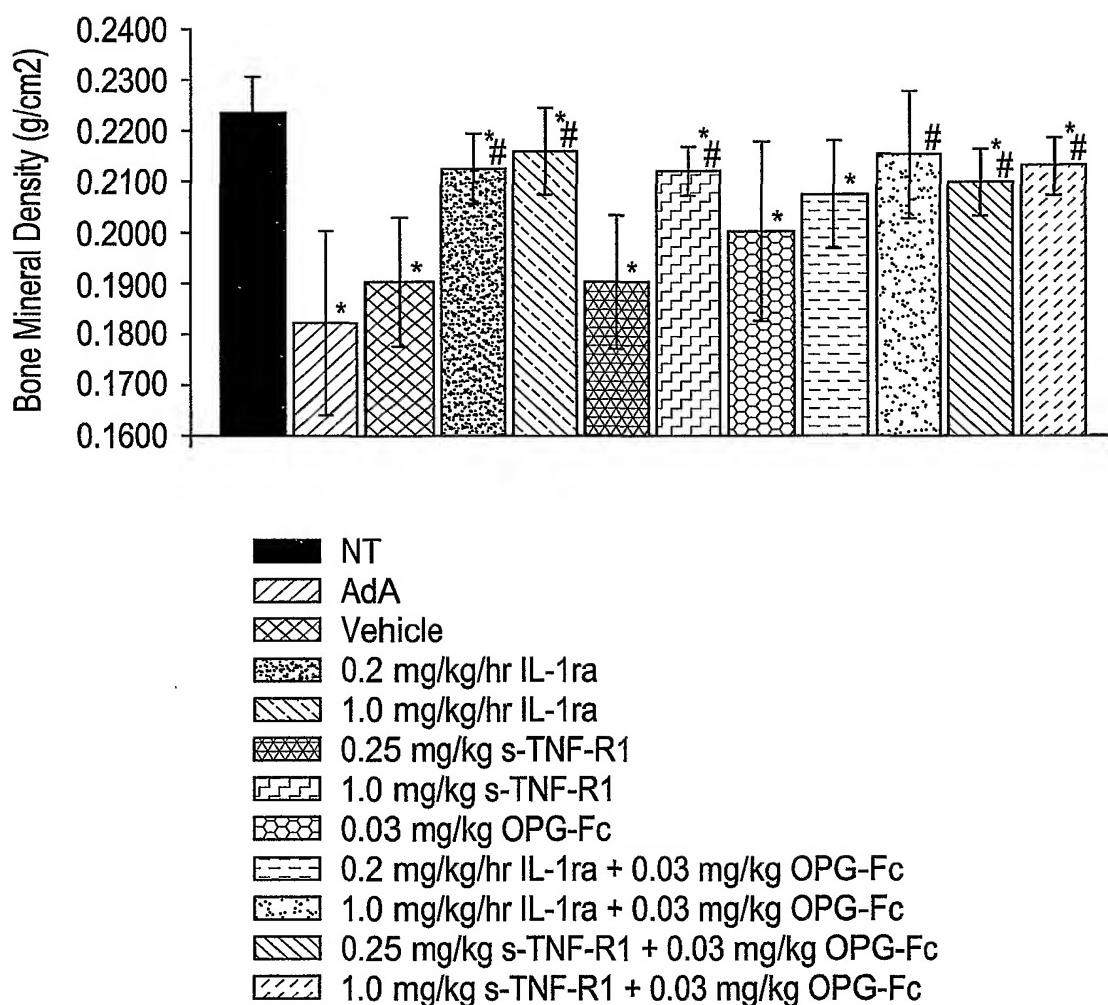
**FIG. 30A-1****FIG. 30A-2**

- ▨ Normal (NT)
- █ Normal + 4 mg/kg OPG-Fc (s.c.)
- ▨ Normal + 1.0 mg/kg OPG-Fc (s.c.)
- ▨ Normal + 0.25 mg/kg OPG-Fc (s.c.)
- ☒ AdA Control
- ▨ AdA + CSEP (2ML1)
- ▨ AdA + OPG Placebo (s.c.)
- ▨ AdA + 4 mg/kg OPG-Fc (s.c.)
- ▨ AdA + 1.0 mg/kg OPG-Fc (s.c.)
- ▨ AdA + 0.25 mg/kg OPG-Fc (s.c.)
- ▨ 15.0 mg/kg/hr IL-1ra (2ML1)
- ☒ 5.0 mg/kg/hr IL-1ra (2ML1)
- ▨ 1.0 mg/kg/hr IL-1ra (2ML1)
- ▨ 0.2 mg/kg/hr IL-1ra (2ML1)
- ▨ AdA + 0.07 mg/kg Dexamethasone (s.c.)

**FIG. 30B-1****FIG. 30B-2**

- ▨ Normal
- █ Normal + 1.0 mg/kg OPG-Fc (s.c.)
- ▨ Normal + 0.25 mg/kg OPG-Fc (s.c.)
- ▨ Normal + 0.0625 mg/kg OPG-Fc (s.c.)
- ▨ Normal + 0.016 mg/kg OPG-Fc (s.c.)
- ▨ Normal + 0.004 mg/kg OPG-Fc (s.c.)
- ▨ AdA control
- ▨ AdA + OPG Placebo (s.c.)
- ▨ AdA + 1.0 mg/kg OPG-Fc (s.c.)
- ▨ AdA + 0.25 mg/kg OPG-Fc (s.c.)
- ▨ AdA + 0.0625 mg/kg OPG-Fc (s.c.)
- ▨ AdA + 0.016 mg/kg OPG-Fc (s.c.)
- ▨ AdA + 0.004 mg/kg OPG-Fc (s.c.)
- ▨ AdA + 5.0 mg/kg/hr IL-1ra (2ML1)
- ▨ AdA + 0.07 mg/kg Dexamethasone (s.c.)

**FIG. 31A**

**FIG. 31B**

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

\*

(i) APPLICANT: Amgen Inc.

(ii) TITLE OF INVENTION: OSTEOPROTEGERIN

(iii) NUMBER OF SEQUENCES: 168

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Amgen Inc.
- (B) STREET: 1840 Dehavilland Drive
- (C) CITY: Thousand Oaks
- (D) STATE: California
- (E) COUNTRY: United States
- (F) ZIP: 91320

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Winter, Robert B.
- (C) REFERENCE/DOCKET NUMBER: A-378-CIP2

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

2

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAAGGAAGGA AAAAAGCGGC CGCTACANNN NNNNNNT

36

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCCACG CGTCCG

16

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTGCGCAG GC

12

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTAAAACGA CGGCCAGT

18

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGGAAACAG CTATGACC

18

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAATTAAACCC TCACTAAAGG

20

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCATTATGAC CCAGAACCG GAC

23

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGTAGCGCC CTTCCCTCAC A TTC 23

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACTAGTCCC ACAATGAACA AGTGGCTGTG 30

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAAGAATGC GGCGCTAAA CTATGAAACA GCCCAGTGAC CATTC 45

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCTCTAGAA AGAGCTGGGA C 21

\*

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCCGTGTTTC CATTATGAG C 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATCAAAGGCA GGGCATACTT CCTG 24

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTGCACTCC TGTTTCACGG TCTG 24

(2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAAGACACCT TGAAGGGCCT GATG

24

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAACTTTAC AGAAGAGCAT CAGC

24

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCGCGGCCG CATGAAACAAG TGGCTGTGCT GCG

33

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTCTAGAG AAACAGCCCA GTGACCATTC C

31

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTGAAGCTGT GCAAGAACCT GATG

24

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCAAAGGCA GGGCATACTT CCTG

24

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGATCCTGA AGCTGCTCAG TTTG

24

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGCGCGGCCG CGGGGACCAAC AATGAACAAG TTG

33

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGCTCTAGAA TTGTGAGGAA ACAGCTCAAT GGC

33

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATAGCGGCCG CTGAGCCAA ATCTTGTGAC AAAACTCAC

39

## (2) INFORMATION FOR SEQ ID NO:25:

<sup>a</sup>

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTAGAGTCG ACTTATCATT TACCCGGAGA CAGGGAGAGG CTCTT

45

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 43 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCTCTGCGGC CGCTAACGAG CTTATTTCA CGGATTGAAC CTG

43

## (2) INFORMATION FOR SEQ ID NO:28:

10

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCCGTAAGAA ACAGCCCCAGT GACC

24

## (2) INFORMATION FOR SEQ ID NO:30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCTCTGCGGC CGCTGTTGCA TTTCCTTCT G

31

## (2) INFORMATION FOR SEQ ID NO:31:

## (i) SEQUENCE CHARACTERISTICS:

11

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Glu	Thr	Leu	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Pro	Glu	Thr	Gly	His
1	5				10			15							

Gln Leu Leu

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTCTGCGGC CGCACACACG TTGTATGTG TTGC

34

(2) INFORMATION FOR SEQ ID NO:34:

12

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TCCCTTGCCC TGACCACTCT T

21

## (2) INFORMATION FOR SEQ ID NO:35:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCTCTGCGGC CGCCTTTGC GTGGCTTCTC TGTT

34

## (2) INFORMATION FOR SEQ ID NO:36:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA.

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

## (2) INFORMATION FOR SEQ ID NO:37:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs

13

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCTCTGCGGC CGCTAAGCAG CTTATTTTA CTGAATGG

38

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 37 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCTCTGCGGC CGCCAGGGTA ACATCTATTC CAC

33

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

14

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CCGAAGCTTC CACCATGAAC AAGTGGCTGT GCTGC

35

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCTCTGTCGA CTATTATAAG CAGCTTATTT TCACGGATTG

40

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCTCTGTCGA CTTAACACAC GTTGTCA<sup>T</sup>TGT GTTGC

35

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TCCCTTGCCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCTCTGTCGA CTTACTTTG CGTGGCTTCT CTGTT

35

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1537 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTGAAGAGCG TGAAGAGCGG TTCCCTCCTT CAGCAAAAA CCCCTCAAGA CCCGTTAGA  
60  
GCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAAT CAGCTTCCTT  
120  
TCGGGCTTTC TTCTCTTCT TCTTCTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC  
180  
CTGCAGGTCTG ACACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCCTCCTT  
240  
TAATTAGTTA AAACAAATCT AGAATCAAAT CGATTAATCG ACTATAACAA ACCATTTCT  
300  
TGC GTAAACC TGTACGATCC TACAGGTACT TATGTTAAC AATTGTATTT CAAGCGATAT  
360  
AATAGTGTGA CAAAAATCCA ATTTATTAGA ATCAAATGTC AATCTATTAC CGTTTTAATG  
420  
ATATATAACA CGCAAAACTT GCGACAAACA ATAGGTAAGG ATAAAGAGAT GGGTATGAAA  
480  
GACATAAATG CCGACGACAC TTACAGAATA ATTAATAAAA TTAAAGCCTG TAGAAGCAAT  
540  
AATGATATTA ATCAATGCTT ATCTGATATG ACTAAAATGG TACATTGTGA ATATTATTTA  
600  
CTCGCGATCA TTTATCCTCA TTCTATGGTT AAATCTGATA TTTCAATTCT GGATAATTAC  
660  
CCTAAAAAAT GGAGGCAATA TTATGATGAC GCTAATTAA TAAAATATGA TCCTATAGTA  
720  
GATTATTCTA ACTCCAATCA TTCACCGATT AATTGGAATA TATTGAAAA CAATGCTGTA  
780  
AATAAAAAT CTCCAAATGT AATTAAAGAA GCGAAATCAT CAGGTCTTAT CACTGGGTTT  
840  
AGTTTCCCTA TTCATACTGC TAATAATGGC TTCCGAATGC TTAGTTTGAC ACATTCAGAG  
900  
AAAGACAACAT ATATAGATAG TTTATTTTA CATGCGTGT A TGAACATACC ATTAATTGTT  
960  
CCTTCTCTAG TTGATAATTA TCGAAAATA AATATAGCAA ATAATAATC AAACAACGAT  
1020

17

TTAACCAAAA GAGAAAAAGA ATGTTTAGCG TGGGCATGCG AAGGAAAAAG CTCTTGGGAT  
1080

ATTCACAAA TATTAGGCTG TAGTAAGCGC ACGGTCACTT TCCATTAAAC CAATGCGCAA.  
1140

ATGAAACTCA ATACAACAAA CCGCTGCCAA AGTATTCTA AAGCAATTAA AACAGGAGCA  
1200

ATTGATTGCC CATACTTAA AAGTTAAGTA CGACGTCCAT ATTTGAATGT ATTTAGAAAA  
1260

ATAAACAAA GAGTTGTAG AAACGCAAAA AGGCCATCCG TCAGGATGGC CTTCTGCTTA  
1320

ATTTGATGCC TGGCAGTTA TGGCGGGCGT CCTGCCCGCC ACCCTCCGGG CCGTTGCTTC  
1380

GCAACGTTCA AATCCGCTCC CGGC GGATTT GTCTACTCA GGAGAGCGTT CACCGACAAA  
1440

CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT TTCGTTTAT TTGATGCCTG  
1500

GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATAC 1537

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAAATTC GGTAC  
55

## (2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 49 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGAATTCCAA CGCGTTAACCC ATATGTTATT CCTCCTTCTA GAATCAAAT

49

## (2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1546 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GCGTAACGTA TGCATGGTCT CCCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA  
60

CGAAAGGCTC AGTCGAAAGA CTGGGCCTT CGTTTTATCT GTTGTGTC GGTGAACGCT  
120

CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCGGA  
180

GGGTGGCGGG CAGGACGCC GCCATAACT GCCAGGCATC AAATTAAGCA GAAGGCCATC  
240

CTGACGGATG GCCTTTTGC GTTTCTACAA ACTCTTTGT TTATTTTCT AAATACATT  
300

AAATATGGAC GTCGTACTTA ACTTTAAAG TATGGGCAAT CAATTGCTCC TGTAAAATT  
360

GCTTAGAAA TACTTGGCA GCGGTTGTT GTATTGAGTT TCATTTGCGC ATTGGTTAAA  
420

TGGAAAGTGA CCGTGCCTT ACTACAGCCT AATATTTTG AAATATCCC AGAGCTTTT  
480

CCTTCGCATG CCCACGCTAA ACATTCTTT TCTCTTTGG TTAAATCGTT GTTGATTAA  
540

TTATTTGCTA TATTATTTT TCGATAATTA TCAACTAGAG AAGGAACAAT TAATGGTATG  
600

TTCATACACG CATGAAAAAA TAAACTATCT ATATAGTTGT CTTTCTCTGA ATGTGCAAAA  
660

CTAACGCATTC CGAACCCATT ATTAGCAGTA TGAATAGGGA AACTAAACCC AGTGATAAGA  
720

CCTGATGATT TCGCTTCTTT AATTACATTT GGAGATTTT TATTTACAGC ATTGTTTCA  
780

AATATATTCC AATTAATCGG TGAATGATTG GAGTTAGAAT AATCTACTAT AGGATCATAT  
840

TTTATTAAAT TAGCGTCATC ATAATATTGC CTCCATTTT TAGGGTAATT ATCCAGAATT  
900

GAAATATCAG ATTTAACCAT AGAATGAGGA TAAATGATCG CGAGTAAATA ATATTCACAA  
960

TGTACCATT TAGTCATATC AGATAAGCAT TGATTAATAT CATTATTGCT TCTACAGGCT  
1020

TTAATTTAT TAATTATTCT GTAAGTGTG TGCGCATTAA TGTCTTCAT ACCCATCTCT  
1080

TTATCCTTAC CTATTGTTG TCGCAAGTTT TGCGTGTAT ATATCATTAA AACGGTAATA  
1140

GATTGACATT TGATTCTAAT AAATTGGATT TTTGTCACAC TATTATATCG CTTGAAATAC  
1200

AATTGTTAA CATAAGTACC TGTAGGATCG TACAGGTTA CGCAAGAAAA TGGTTGTTA  
1260

TAGTCGATTA ATCGATTGA TTCTAGATT GTTTAACTA ATTAAAGGAG GAATAACATA  
1320

20

TGGTTAACGC GTTGAATTG GAGCTCACTA GTGTCGACCT GCAGGGTACC ATGGAAGCTT  
1380

A<sup>C</sup>TCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC CCGAAAGGAA GCTGAGTTGG  
1440

CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG GGCCTCTAAA CGGGTCTTGA  
1500

GGGGTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT TCACGC 1546

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 47 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATGAAACAT CATCACCATC ACCATCATGC TAGCGTTAAC GCGTTGG 47

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AATTCCAACG CGTTAACGCT AGCATGATGG TGATGGTGAT GATGTTCA 49

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 141 base pairs
- (B) TYPE: nucleic acid

21

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

\*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CTAATTCCGC TCTCACCTAC CAAACAATGC CCCCCCTGCAA AAAATAAATT CATATAAAAA  
60

ACATACAGAT AACCATCTGC GGTGATAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC  
120

TGGCGGTGAT ACTGAGCACA T

141

(2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 147 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGATGTGCTC AGTATCACCG CCAGTGGTAT TTATGTCAAC ACCGCCAGAG ATAATTTATC  
60

ACCGCAGATG GTTATCTGTA TGTTTTTAT ATGAATTAT TTTTGAGG GGGGCATTGT  
120

TTGGTAGGTG AGAGCGGAAT TAGACGT

147

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 55 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

22

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAAATTG GGTAC  
53.

## (2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 49 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGAATTCCAA CGCGTTAACCC ATATGTTATT CCTCCTTCTA GAATCAAAT

49.

## (2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 668 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GTAAGAGAGCG TGAAGAGCGG TTCCCTCCTT CAGCAAAAAA CCCCTCAAGA CCCGTTAGA  
60

GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAAGT CAGCTTCCTT  
120

TGGGGCTTTC TTCTTCTTCT TCTTCTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC  
180

CTGCAGGTCTG ACACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCCTCCTT  
240

TAATTAGTTA ACTCAAATCT AGAATCAAAT CGATAAATTG TGAGCGCTCA CAATTGAGAA  
300

TATTAATCAA GAATTTAGC ATTTGTCAA TGAATTTTT AAAAATTATG AGACGTCCAT  
360

ATTTGAATGT ATTTAGAAAA ATAAACAAAA GAGTTGTAG AAACGCAAAA AGGCCATCCG  
420

TCAGGATGGC CTTCTGCTTA ATTTGATGCC TGGCAGTTA TGGCGGGCGT CCTGCCCGCC  
480

ACCTCCGGG CCGTTGCTTC GCAACGTTCA AATCCGCTCC CGGC GGATTT GTCCTACTCA  
540

GGAGAGCGTT CACCGACAAA CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT  
600

TCGTTTTAT TTGATGCCTG GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATACGTT  
660

ACGCACGT 668

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 726 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GCGTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA  
60

CGAAAGGCTC AGTCGAAAGA CTGGGCCTT CGTTTTATCT GTTGTGTC GGTGAACGCT  
120

CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCGGA  
180

GGGTGGCGGG CAGGACGCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGGGCCT  
240

CCCACCGCCC GTCCTGCGGG CGGTATTGGA CGGTCCGTAG TTTAATTCTGT CTTCGCCATC  
300

24

CTGACGGATG GCCTTTTGC GTTTCTACAA ACTCTTTGT TTATTTTCT AAATACATTC  
360

AAATATGGAC GTCTCATAAT TTTTAAAAAA TTCATTTGAC AAATGCTAAA ATTCTTGATT  
420

AATATTCTCA ATTGTGAGCG CTCACAATTT ATCGATTTGA TTCTAGATTT GTTTAACTA  
480

ATTAAAGGAG GAATAACATA TGGTTAACGC GTTGGAAATTC GAGCTCACTA GTGTCGACCT  
540

GCAGGGTACC ATGGAAGCTT ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC  
600

CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG  
660

GCGCTCTAAA CGGGTCTTGA GGGTTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT  
720

TCACGC

726

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT GGAC

44

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GTCCTCCTGG TACCTACCTA AAACAAC

27

(2) INFORMATION FOR SEQ ID NO:61:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 102 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTACCCGGCG  
60

GACATTTATC ACACAGCAGC TGATGAGAAG TTTCTTCATC CA

102

(2) INFORMATION FOR SEQ ID NO:62:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Met Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro  
1 5 10 15

Gly Thr Tyr

(2) INFORMATION FOR SEQ ID NO:63:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 84 base pairs
  - (B) TYPE: nucleic acid

26

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATGGAAACT TTTCTTCAA AATATCTTCA TTATGATGAA GAAACTTCTC ATCAGCTGCT  
60

GTGTGATAAA TGTCCGCCGG GTAC

84

(2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 78 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCATCAA TGAAGATATT  
60

TTGGAGGAAA AGTTCCA

78

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 44 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TACGCACGGG ATCCTTATAA GCAGCTTATT TTCACGGATT GAAC

44

(2) INFORMATION FOR SEQ ID NO:66:

27

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

GTGCTCCTGG TACCTACCTA AACACAGCACT GCACAGTG

38

## (2) INFORMATION FOR SEQ ID NO:67:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATGGAAACT CTGCCTCCAA AATACTGCA TTACGATCCG GAAACTGGTC ATCAGCTGCT  
60

GTGTGATAAA TGTGCTCCGG GTAC

84

## (2) INFORMATION FOR SEQ ID NO:68:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT  
60

TTGGAGGCAG AGTTCCA

78

## (2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TATGGACCCA GAAACTGGTC ATCAGCTGCT GTGTGATAAA TGTGCTCCGG GTAC  
54

## (2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 48 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC TGGGTCCA

48

## (2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 87 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

29

TATGAAAGAA ACTCTGCCTC CAAAATACCT GCATTACGAT CGGGAAACTG GTCATCAGCT  
60

GCTGTGTGAT AAATGTGCTC CGGGTAC 87

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 81 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CCGGAGCAC A TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT  
60

TTGGAGGCAG AGTTTCTTTC A 81

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GTTCTCCTCA TATGAAACAT CATCACCATC ACCATCATGA AACTCTGCCT CCAAAATACC  
60

TGCATTACGA T 71

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid

30

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GTTCTCCTCA TATGAAAGAA ACTCTGCCTC CAAAATACCT GCA

43

(2) INFORMATION FOR SEQ ID NO:75:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 76 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TACGCACTGG ATCCTTAATG ATGGTGATGG TGATGATGTA AGCAGCTTAT TTTCACGGAT  
60

TGAACCTGAT TCCCTA

76

(2) INFORMATION FOR SEQ ID NO:76:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 47 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GTTCTCCTCA TATGAAATAC CTGCATTACG ATCCGGAAAC TGGTCAT

47

(2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 43 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

31

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

R

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GTTCTCCTAT TAATGAAATA TCTTCATTAT GATGAAGAAA CTT

43

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

TACGCACGGG ATCCTTATAA GCAGCTTATT TTTACTGATT

40

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

GTTCTCCTCA TATGGAAACT CTGCCTCCAA AATACCTGCA

40

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

32

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TACGGCACTGG ATCCTTATGT TGCATTCCT TTCTGAATTA GCA

43

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CCGGAAACAG ATAATGAG

18

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GATCCTCATT ATCTGTTT

18

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCGGAAACAG AGAAGCCACG CAAAAGTAAG

30

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

GATCCTTACT TTTGCGTGCG TTCTCTGTTT

30

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATGTTAACATG AG

12

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

34

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GATCCTCATT AACAA 14

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

TATGTTCCGG AAACAGTTAA G 21

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

GATCCTTAAC TGTTTCCGGA ACA 23

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

35

TATGTTCCGG AACAGTGAA TCAACTAAA AATAAG

36

## (2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GATCCTTATT TTTGAGTTGA TTCACTGTTT CCGGAACA

38

## (2) INFORMATION FOR SEQ ID NO:91:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 100 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

CTAGCGACGA CGACGACAAA GAAACTCTGC CTCCAAAATA CCTGCATTAC GATCCGGAAA  
60

CTGGTCATCA GCTGCTGTGT GATAAATGTG CTCCGGGTAC

100

## (2) INFORMATION FOR SEQ ID NO:92:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 92 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

36

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT  
60

TTGGAGGCAG AGTTTCTTG TCGTCGTCGT CG

92

(2) INFORMATION FOR SEQ ID NO:93:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ACAAACACAA TCGATTTGAT ACTAGA

26

(2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TTTGTAA CTAATTAAG GAGGAATAAA ATATGAGAGG ATCGCATCAC

50

(2) INFORMATION FOR SEQ ID NO:95:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

CAT~~C~~ACCATC ACGAACCTT CCCGCCGAAA TACCTGCACT ACGACGAAGA

50

## (2) INFORMATION FOR SEQ ID NO:96:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

AACCTCCCAC CAGCTGCTGT GCGACAAATG CCCGCCGGGT ACCCAAACA

49

## (2) INFORMATION FOR SEQ ID NO:97:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

TGTGGGGTA CCCGGCGGGC ATTTGT

26

## (2) INFORMATION FOR SEQ ID NO:98:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

38

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CGCACAGCAG CTGGTGGGAG GTTTCTTCGT CGTAGTGCAG GTATTCGGC

50

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GGGAAGGTTT CGTGATGGTG ATGGTGATGC GATCCTCTCA TATTTTATT

49

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

CCTCCTTTAA TTAGTTAAAA CAAATCTAGT ATCAAATCGA TTGTGTTGT

50

(2) INFORMATION FOR SEQ ID NO:101:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

39

ACAAACACAA TCGATTTGAT ACTAGATTTG TTTTAACCAA TTAAAGGAGG AATAAAATG  
59

## (2) INFORMATION FOR SEQ ID NO:102:

~

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 48 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

CTAATTAAAG GAGGAATAAA ATGAAAGAAA CTTTCCTCC AAAATATC

48

## (2) INFORMATION FOR SEQ ID NO:103:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C

31

## (2) INFORMATION FOR SEQ ID NO:104:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 59 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

40

ACAAACACAA TCGATTTGAT ACTAGATTG TTTTAACCAA TTAAAGGAGG AATAAAATG  
59

## (2) INFORMATION FOR SEQ ID NO:105:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

CTAATTAAAG GAGGAATAAA ATGAAAAAAA AAGAAACTTT TCCTCCAAAAA TATC  
54

## (2) INFORMATION FOR SEQ ID NO:106:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C

31

## (2) INFORMATION FOR SEQ ID NO:107:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

41

CAGCCC GG GT AAAATGGAAA CGTTT CCTCC AAAATATCTT CATT

44

## (2) INFORMATION FOR SEQ ID NO:108:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

CGTTTCCATT TTACCCGGGC TGAGCGAGAG GCTCTTCTGC GTGT

44

## (2) INFORMATION FOR SEQ ID NO:109:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

CGCTCAGCCC GGGTAAATG GAAACGTTGC CTCCAAAATA CCTGC

45

## (2) INFORMATION FOR SEQ ID NO:110:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

CCATTTTACC CGGGCTGAGC GAGAGGCTCT TCTGCGTGT

39

42

## (2) INFORMATION FOR SEQ ID NO:111:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

GAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

## (2) INFORMATION FOR SEQ ID NO:112:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

CAGCTGCAGC TAAGCAGCTT ATTTTCACGG ATTG

34

## (2) INFORMATION FOR SEQ ID NO:113:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

AAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

## (2) INFORMATION FOR SEQ ID NO:114:

43

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

CAGCTGCAGC TAAGCAGCTT ATTTTTACTG ATTGG

35

## (2) INFORMATION FOR SEQ ID NO:115:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

CTAGAAGGAG GAATAACATA TGGAAACTTT TGCTCCAAAA TATCTTCATT ATGATGAAGA  
60

AACTAGTCAT CAGCTGCTGT GTGATAAAATG TCCGCCGGGT AC

102

## (2) INFORMATION FOR SEQ ID NO:116:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 94 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

44

CCGGCGGACA TTTATCACAC AGCAGCTGAT GACTAGTTTC TTCATCATAA TGAAGATATT  
60

TTGGAGCAAA AGTTTCCATA TGTTATTCCCT CCTT

94

(2) INFORMATION FOR SEQ ID NO:117:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

CTAGAAGGAG GAATAACATA TGGAAACTTT TCCTGCTAAA TATCTTCATT ATGATGAAGA  
60

AA

62

(2) INFORMATION FOR SEQ ID NO:118:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

CTAGTTCTT CATCATAATG AAGATATTTA GCAGGAAAAG TTTCCATATG TTATTCCCTCC  
60

TT

62

(2) INFORMATION FOR SEQ ID NO:119:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: protein

R.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

Tyr His Tyr Tyr Asp Gln Asn Gly Arg Met Cys Glu Glu Cys His Met  
1 5 10 15

Cys Gln Pro Gly His Phe Leu Val Lys His Cys Lys Gln Pro Lys Arg  
20 25 30

Asp Thr Val Cys His Lys Pro Cys Glu Pro Gly Val Thr Tyr Thr Asp  
35 40 45

Asp Trp His  
50

(2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2432 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 124..1326

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

ATCAAAGGCA GGGCATACTT CCTGTTGCC AGACCTTATA TAAAACGTCA TGTCGCCTG  
60

GGCAGCAGAG AAGCACCTAG CACTGGCCCA GC GGCTGCCG CCTGAGGTTT CCAGAGGACC  
120

ACA ATG AAC AAG TGG CTG TGC TGT GCA CTC CTG GTG TTC TTG GAC ATC  
168

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile  
1 5 10 15

ATT GAA TGG ACA ACC CAG GAA ACC TTT CCT CCA AAA TAC TTG CAT TAT  
216

Ile Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr  
20            25            30

\*

GAC CCA GAA ACC GGA CGT CAG CTC TTG TGT GAC AAA TGT GCT CCT GGC  
264

Asp Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly  
35            40            45

ACC TAC CTA AAA CAG CAC TGC ACA GTC AGG AGG AAG ACA CTG TGT GTC  
312

Thr Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val  
50            55            60

CCT TGC CCT GAC TAC TCT TAT ACA GAC AGC TGG CAC ACG AGT GAT GAA  
360

Pro Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu  
65            70            75

TGC GTG TAC TGC AGC CCC GTG TGC AAG GAA CTG CAG ACC GTG AAA CAG  
408

Cys Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln  
80            85            90            95

GAG TGC AAC CGC ACC CAC AAC CGA GTG TGC GAA TGT GAG GAA GGG CGC  
456

Glu Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg  
100            105            110

TAC CTG GAG CTC GAA TTC TGC TTG AAG CAC CGG AGC TGT CCC CCA GGC  
504

Tyr Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly  
115            120            125

TTG GGT GTG CTG CAG GCT GGG ACC CCA GAG CGA AAC ACG GTT TGC AAA  
552

Leu Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys  
130            135            140

AGA TGT CCG GAT GGG TTC TTC TCA GGT GAG ACG TCA TCG AAA GCA CCC  
600

Arg Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro  
145            150            155

TGT AGG AAA CAC ACC AAC TGC AGC TCA CTT GGC CTC CTG CTA ATT CAG  
648

Cys Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln  
160            165            170            175

AAA GGA AAT GCA ACA CAT GAC AAT GTA TGT TCC GGA AAC AGA GAA GCA  
 696  
 Lys Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala  
 \* 180 185 190  
  
 ACT CAA AAT TGT GGA ATA GAT GTC ACC CTG TGC GAA GAG GCA TTC TTC  
 744  
 Thr Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe  
 195 200 205  
  
 AGG TTT GCT GTG CCT ACC AAG ATT ATA CCG AAT TGG CTG AGT GTT CTG  
 792  
 Arg Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu  
 210 215 220  
  
 GTG GAC AGT TTG CCT GGG ACC AAA GTG AAT GCA GAG AGT GTA GAG AGG  
 840  
 Val Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg  
 225 230 235  
  
 ATA AAA CGG AGA CAC AGC TCG CAA GAG CAA ACT TTC CAG CTA CTT AAG  
 888  
 Ile Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys  
 240 245 250 255  
  
 CTG TGG AAG CAT CAA AAC AGA GAC CAG GAA ATG GTG AAG AAG ATC ATC  
 936  
 Leu Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile  
 260 265 270  
  
 CAA GAC ATT GAC CTC TGT GAA AGC AGT GTG CAA CGG CAT ATC GGC CAC  
 984  
 Gln Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His  
 275 280 285  
  
 GCG AAC CTC ACC ACA GAG CAG CTC CGC ATC TTG ATG GAG AGC TTG CCT  
 1032  
 Ala Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro  
 290 295 300  
  
 GGG AAG AAG ATC AGC CCA GAC GAG ATT GAG AGA ACG AGA AAG ACC TGC  
 1080  
 Gly Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys  
 305 310 315  
  
 AAA CCC AGC GAG CAG CTC CTG AAG CTA CTG AGC TTG TGG AGG ATC AAA  
 1128  
 Lys Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys  
 320 325 330 335  
  
 AAT GGA GAC CAA GAC ACC TTG AAG GGC CTG ATG TAC GCA CTC AAG CAC  
 1176  
 Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His

48

340 345 350

TTG AAA GCA TAC CAC TTT CCC AAA ACC GTC ACC CAC AGT CTG AGG AAG  
1224  
Leu Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys  
355 360 365

ACC ATC AGG TTC TTG CAC AGC TTC ACC ATG TAC CGA TTG TAT CAG AAA  
1272  
Thr Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys  
370 375 380

CTC TTT CTA GAA ATG ATA GGG AAT CAG GTT CAA TCA GTG AAG ATA AGC  
1320  
Leu Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser  
385 390 395

TGC TTA TAGTTAGGAA TGGTCACTGG GCTGTTCTT CAGGATGGGC CAACACTGAT  
1376  
Cys Leu  
400

GGAGCAGATG GCTGCTTCTC CGGCTCTGA AATGGCAGTT GATTCCCTTC TCATCAGTTG  
1436

GTGGGAATGA AGATCCTCCA GCCCAACACA CACACTGGGG AGTCTGAGTC AGGAGAGTGA  
1496

GGCAGGCTAT TTGATAATTG TGCAAAGCTG CCAGGTGTAC ACCTAGAAAG TCAAGCACCC  
1556

TGAGAAAGAG GATATTTTA TAACCTCAA CATAGGCCCT TTCCTTCCTC TCCTTATGGA  
1616

TGAGTACTCA GAAGGCTTCT ACTATCTTCT GTGTCATCCC TAGATGAAGG CCTCTTTAT  
1676

TTATTTTTT ATTCTTTTT TC GGAGCTGG GGACCGAAC CAGGGCCTTG CGCTTGCGAG  
1736

GCAAGTGCTC TACCACTGAG CTAAATCTCC AACCCCTGAA GGCCTCTTC TTTCTGCCTC  
1796

TGATAGTCTA TGACATTCTT TTTCTACAA TT CGTATCAG GTGCACGAGC CTTATCCCAT  
1856

TTGTAGGTTT CTAGGCAAGT TGACCGTTAG CTATTTTCC CTCTGAAGAT TTGATTGAG  
1916

TTGCAGACTT GGCTAGACAA GCAGGGTAG GTTATGGTAG TTTATTTAAC AGACTGCCAC  
1976

CAGGAGTCCA GTGTTCTTG TTCCCTGT A GTTGTACCTA AGCTGACTCC AAGTACATTT  
2036

AGTATGAAAA ATAATCAACA AATTTTATTC CTTCTATCAA CATTGGCTAG CTTTGTTC  
2096

GGGCACTAAA AGAAACTACT ATATGGAGAA AGAATTGATA TTGCCCCCAA CGTCAACAA  
2156

CCCAATAGTT TATCCAGCTG TCATGCCTGG TTCAGTGTCT ACTGACTATG CGCCCTCTTA  
2216

TTACTGCATG CAGTAATTCA ACTGGAAATA GTAATAATAA TAATAGAAAT AAAATCTAGA  
2276

CTCCATTGGA TCTCTCTGAA TATGGGAATA TCTAACTTAA GAAGCTTGA GATTCAGTT  
2336

GTTGTTAAAGG CTTTTATTAA AAAGCTGATG CTCTTCTGT A AAAGTTACTA ATATATCTGT  
2396

AAGACTATT A CAGTATTGCT ATTTATATCC ATCCAG 2432

(2) INFORMATION FOR SEQ ID NO:121:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 401 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Met	Asn	Lys	Trp	Leu	Cys	Cys	Ala	Leu	Leu	Val	Phe	Leu	Asp	Ile	Ile
1	5			10	.				15						

Glu	Trp	Thr	Thr	Gln	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp
				20	25				30						

Pro	Glu	Thr	Gly	Arg	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	Thr
				35	40			45							

Tyr	Leu	Lys	Gln	His	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro
				50	55		60								

Cys	Pro	Asp	Tyr	Ser	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys
				65	70		75		80						

50

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu  
85 90 95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Gly Arg Tyr  
100 105 110

Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu  
115 120 125

Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg  
130 135 140

Cys Pro Asp Gly Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln Lys  
165 170 175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr  
180 185 190

Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
195 200 205

Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val  
210 215 220

Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile  
225 230 235 240

Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu  
245 250 255

Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Ile Ile Gln  
260 265 270

Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His Ala  
275 280 285

Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro Gly  
290 295 300

51

ys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys  
305            310            315            320

Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn  
`            325            330            335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu  
340            345            350

Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr  
355            360            365

Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu  
370            375            380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
385            390            395            400

Leu

(2) INFORMATION FOR SEQ ID NO:122:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1324 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 90..1292

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

CCTTATATAAA ACGTCATGAT TGCCTGGGCT GCAGAGACGC ACCTAGCACT GACCCAGCGG  
60

CTGCCTCCTG AGGTTTCCCG AGGACCACA ATG AAC AAG TGG CTG TGC TGC GCA  
113

Met Asn Lys Trp Leu Cys Cys Ala  
1            5

CTC CTG GTG CTC CTG GAC ATC ATT GAA TGG ACA ACC CAG GAA ACC CTT  
161

Leu Leu Val Leu Leu Asp Ile Ile Glu Trp Thr Thr Gln Glu Thr Leu  
10            15            20

CCT CCA AAG TAC TTG CAT TAT GAC CCA GAA ACT GGT CAT CAG CTC CTG  
209  
Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His Gln Leu Leu  
25           30           35           40

TGT GAC AAA TGT GCT CCT GGC ACC TAC CTA AAA CAG CAC TGC ACA GTG  
257  
Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His Cys Thr Val  
45           50           55

AGG AGG AAG ACA TTG TGT GTC CCT TGC CCT GAC CAC TCT TAT ACG GAC  
305  
Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser Tyr Thr Asp  
60           65           70

AGC TGG CAC ACC AGT GAT GAG TGT GTG TAT TGC AGC CCA GTG TGC AAG  
353  
Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro Val Cys Lys  
75           80           85

GAA CTG CAG TCC GTG AAG CAG GAG TGC AAC CGC ACC CAC AAC CGA GTG  
401  
Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val  
90           95           100

TGT GAG TGT GAG GAA GGG CGT TAC CTG GAG ATC GAA TTC TGC TTG AAG  
449  
Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys  
105           110           115           120

CAC CGG AGC TGT CCC CCG GGC TCC GGC GTG GTG CAA GCT GGA ACC CCA  
497  
His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala Gly Thr Pro  
125           130           135

GAG CGA AAC ACA GTT TGC AAA AAA TGT CCA GAT GGG TTC TTC TCA GGT  
545  
Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe Phe Ser Gly  
140           145           150

GAG ACT TCA TCG AAA GCA CCC TGT ATA AAA CAC ACG AAC TGC AGC ACA  
593  
Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn Cys Ser Thr  
155           160           165

TTT GGC CTC CTG CTA ATT CAG AAA GGA AAT GCA ACA CAT GAC AAC GTG  
641  
Phe Gly Leu Leu Leu Ile Gln Lys Gly Asn Ala Thr His Asp Asn Val  
170           175           180

TGT TCC GGA AAC AGA GAA GCC ACG CAA AAG TGT GGA ATA GAT GTC ACC  
689  
Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile Asp Val Thr

53

185            190            195            200  
CTG TGT GAA GAG GCC TTC TTC AGG TTT GCT GTT CCT ACC AAG ATT ATA  
737  
Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Ile Ile  
205            210            215  
  
CCA AAT TGG CTG AGT GTT TTG GTG GAC AGT TTG CCT GGG ACC AAA GTG  
785  
Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly Thr Lys Val  
220            225            230  
  
AAT GCC GAG AGT GTA GAG AGG ATA AAA CGG AGA CAC AGC TCA CAA GAG  
833  
Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser Ser Gln Glu  
235            240            245  
  
CAA ACC TTC CAG CTG CTG AAG CTG TGG AAA CAT CAA AAC AGA GAC CAG  
881  
Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Arg Asp Gln  
250            255            260  
  
GAA ATG GTG AAG AAG ATC ATC CAA GAC ATT GAC CTC TGT GAA AGC AGC  
929  
Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Ser Ser  
265            270            275            280  
  
GTG CAG CGG CAT CTC GGC CAC TCG AAC CTC ACC ACA GAG CAG CTT CTT  
977  
Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu Gln Leu Leu  
285            290            295  
  
GCC TTG ATG GAG AGC CTG CCT GGG AAG AAG ATC AGC CCA GAA GAG ATT  
1025  
Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro Glu Glu Ile  
300            305            310  
  
GAG AGA ACG AGA AAG ACC TGC AAA TCG AGC GAG CAG CTC CTG AAG CTA  
1073  
Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu Leu Lys Leu  
315            320            325  
  
CTC AGT TTA TGG AGG ATC AAA AAT GGT GAC CAA GAC ACC TTG AAG GGC  
1121  
Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly  
330            335            340  
  
CTG ATG TAT GCC CTC AAG CAC TTG AAA ACA TCC CAC TTT CCC AAA ACT  
1169  
Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe Pro Lys Thr  
345            350            355            360

54

GTC ACC CAC AGT CTG AGG AAG ACC ATG AGG TTC CTG CAC AGC TTC ACA  
1217

Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His Ser Phe Thr  
365            370            375

ATG TAC AGA CTG TAT CAG AAG CTC TTT TTA GAA ATG ATA GGG AAT CAG  
1265

Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn Gln  
380            385            390

GTT CAA TCC GTG AAA ATA AGC TGC TTA TAACTAGGAA TGGTCACTGG  
1312

Val Gln Ser Val Lys Ile Ser Cys Leu  
395            400

GCTGTTTCTT CA

1324

(2) INFORMATION FOR SEQ ID NO:123:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 401 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Leu Leu Asp Ile Ile  
1            5            10            15

Glu Trp Thr Thr Gln Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp  
20            25            30

Pro Glu Thr Gly His Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr  
35            40            45

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro  
50            55            60

Cys Pro Asp His Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys  
65            70            75            80

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu  
85            90            95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr  
100            105            110

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser

55

115 120 125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys  
130 135 140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Ile Lys His Thr Asn Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys  
165 170 175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr  
180 185 190

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
195 200 205

Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val  
210 215 220

Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile  
225 230 235 240

Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu  
245 250 255

Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Ile Ile Gln  
260 265 270

Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Leu Gly His Ser  
275 280 285

Asn Leu Thr Thr Glu Gln Leu Ala Leu Met Glu Ser Leu Pro Gly  
290 295 300

Lys Lys Ile Ser Pro Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys  
305 310 315 320

Ser Ser Glu Gln Leu Leu Lys Leu Ser Leu Trp Arg Ile Lys Asn  
325 330 335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu  
340 345 350

Lys Thr Ser His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr  
355 360 365

Met Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu  
370 375 380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
385 390 395 400

Leu

## (2) INFORMATION FOR SEQ ID NO:124:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1355 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 94..1296

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

GTATATATAA CGTGATGAGC GTACGGGTGC GGAGACGCAC CGGAGCGCTC GCCCAGCCGC  
60

CGCTCCAAGC CCCTGAGGTT TCCGGGGACC ACA ATG AAC AAG TTG CTG TGC TGC  
114

Met Asn Lys Leu Leu Cys Cys  
1 5

GCG CTC GTG TTT CTG GAC ATC TCC ATT AAG TGG ACC ACC CAG GAA ACG  
162

Ala Leu Val Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr  
10 15 20

TTT CCT CCA AAG TAC CTT CAT TAT GAC GAA GAA ACC TCT CAT CAG CTG  
210

Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His Gln Leu  
25 30 35

TTG TGT GAC AAA TGT CCT CCT GGT ACC TAC CTA AAA CAA CAC TGT ACA  
258

Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His Cys Thr  
40 45 50 55

GCA AAG TGG AAG ACC GTG TGC GCC CCT TGC CCT GAC CAC TAC TAC ACA  
306

Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr  
60 65 70

GAC AGC TGG CAC ACC AGT GAC GAG TGT CTA TAC TGC AGC CCC GTG TGC  
354

Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys

57

75            80            85

AAG GAG CTG CAG TAC GTC AAG CAG GAG TGC AAT CGC ACC CAC AAC CGC  
402  
Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg  
90            95            100

GTG TGC GAA TGC AAG GAA GGG CGC TAC CTT GAG ATA GAG TTC TGC TTG  
450  
Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu  
105            110            115

AAA CAT AGG AGC TGC CCT CCT GGA TTT GGA GTG GTG CAA GCT GGA ACC  
498  
Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr  
120            125            130            135

CCA GAG CGA AAT ACA GTT TGC AAA AGA TGT CCA GAT GGG TTC TTC TCA  
546  
Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe Ser  
140            145            150

AAT GAG ACG TCA TCT AAA GCA CCC TGT AGA AAA CAC ACA AAT TGC AGT  
594  
Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn Cys Ser  
155            160            165

GTC TTT GGT CTC CTG CTA ACT CAG AAA GGA AAT GCA ACA CAC GAC AAC  
642  
Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His Asp Asn  
170            175            180

ATA TGT TCC GGA AAC AGT GAA TCA ACT CAA AAA TGT GGA ATA GAT GTT  
690  
Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile Asp Val  
185            190            195

ACC CTG TGT GAG GAG GCA TTC TTC AGG TTT GCT GTT CCT ACA AAG TTT  
738  
Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Phe  
200            205            210            215

ACG CCT AAC TGG CTT AGT GTC TTG GTA GAC AAT TTG CCT GGC ACC AAA  
786  
Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly Thr Lys  
220            225            230

GTA AAC GCA GAG AGT GTA GAG AGG ATA AAA CGG CAA CAC AGC TCA CAA  
834  
Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser Ser Gln  
235            240            245

58

GAA CAG ACT TTC CAG CTG CTG AAG TTA TGG AAA CAT CAA AAC AAA GCC  
 882

Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Lys Ala  
 250 255 260

CAA GAT ATA GTC AAG AAG ATC ATC CAA GAT ATT GAC CTC TGT GAA AAC  
 930

Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Asn  
 265 270 275

AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC TTC GAG CAG CTT  
 978

Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu Gln Leu  
 280 285 290 295

CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG GGA GCA GAA GAC  
 1026

Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp  
 300 305 310

ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC CAG ATC CTG AAG  
 1074

Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys  
 315 320 325

CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA GAC ACC TTG AAG  
 1122

Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys  
 330 335 340

GGC CTA ATG CAC GCA CTA AAG CAC TCA AAG ACG TAC CAC TTT CCC AAA  
 1170

Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys  
 345 350 355

ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC CTT CAC AGC TTC  
 1218

Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe  
 360 365 370 375

ACA ATG TAC AAA TTG TAT CAG AAG TTA TTT TTA GAA ATG ATA GGT AAC  
 1266

Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn  
 380 385 390

CAG GTC CAA TCA GTA AAA ATA AGC TGC TTA TAACTGGAAA TGGCCATTGA  
 1316

Gln Val Gln Ser Val Lys Ile Ser Cys Leu  
 395 400

GCTGTTTCCT CACAATTGGC GAGATCCCAT GGATGATAA

1355

## (2) INFORMATION FOR SEQ ID NO:125:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 401 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

Met Asn Lys Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile  
1 5 10 15

Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp  
20 25 30

Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr  
35 40 45

Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro  
50 55 60

Cys Pro Asp His Tyr Tyr Asp Ser Trp His Thr Ser Asp Glu Cys  
65 70 75 80

Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu  
85 90 95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr  
100 105 110

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe  
115 120 125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg  
130 135 140

Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys  
165 170 175

Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr  
180 185 190

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
195 200 205

Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val  
210 215 220

60

Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile  
225            230            235            240

Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu  
\*            245            250            255

Trp Lys His Gln Asn Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln  
260            265            270

Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala  
275            280            285

Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly  
290            295            300

Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys  
305            310            315            320

Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn  
325            330            335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser  
340            345            350

Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr  
355            360            365

Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu  
370            375            380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
385            390            395            400

Leu

(2) INFORMATION FOR SEQ ID NO:126:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 139 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys  
1            5            10            15

61

Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro  
20 25 30

Gly Gln Asp Thr Asp Cys Arg Glu Cys Ser Gly Ser Phe Thr Ala  
\* 35 40 45

Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys  
50 55 60

Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr  
65 70 75 80

Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn  
85 90 95

Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His  
100 105 110

Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly  
115 120 125

Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys  
130 135

## (2) INFORMATION FOR SEQ ID NO:127:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 48 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

## (2) INFORMATION FOR SEQ ID NO:128:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 219 amino acids

(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala  
1 5 10 15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser  
20 25 30

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Val Glu Thr Gln Asn  
35 40 45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro  
50 55 60

Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro  
65 70 75 80

Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His  
85 90 95

Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly  
100 105 110

Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg  
115 120 125

Cys Lys Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp  
130 135 140

Pro Cys Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr  
145 150 155 160

Ser Asn Thr Lys Cys Lys Glu Glu Gly Ser Arg Ser Asn Leu Gly Trp  
165 170 175

Leu Cys Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg  
180 185 190

Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly  
195 200 205

Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr  
210 215

## (2) INFORMATION FOR SEQ ID NO:129:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 280 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu  
1           5           10           15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro  
20           25           30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys  
35           40           45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys  
50           55           60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp  
65           70           75           80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu  
85           90           95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val  
100          105          110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg  
115          120          125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe  
130          135          140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu  
145          150          155          160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu  
165          170          175

Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr  
180          185          190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser  
195          200          205

64

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu  
210                215                220

Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys  
225                230                235                240

\*  
Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu  
245                250                255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser  
260                265                270

Phe Ser Pro Thr Pro Gly Phe Thr  
275                280

## (2) INFORMATION FOR SEQ ID NO:130:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 207 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr Val Tyr Gly  
1                5                10                15

Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly Gly His Asp  
20                25                30

Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro Gly Phe Tyr  
35                40                45

Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys Ser Pro Cys  
50                55                60

Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val  
65                70                75                80

Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser Gln Pro Cys  
85                90                95

65

Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly Asn Tyr Cys  
 100 105 110

Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro Gln Thr Lys  
 115 120 125

Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala Gly Asp Thr  
 130 135 140

Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser Leu Ser Pro  
 145 150 155 160

Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val Gly Phe Asn  
 165 170 175

Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Thr Ala Gly His Asn  
 180 185 190

Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn Tyr Thr  
 195 200 205

## (2) INFORMATION FOR SEQ ID NO:131:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 227 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

Met ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu  
 1 5 10 15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr  
 20 25 30

Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln  
 35 40 45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys  
 50 55 60

Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp  
 65 70 75 80

Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys  
 85 90 95

Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg  
100 105 110

Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu  
115 120 125

Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg  
130 135 140

Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val  
145 150 155 160

Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr  
165 170 175

Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly  
180 185 190

Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser  
195 200 205

Met ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser  
210 215 220

Gln His Thr  
225

## (2) INFORMATION FOR SEQ ID NO:132:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 197 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

Met Val Ser Leu Pro Arg Leu Cys Ala Leu Trp Gly Cys Leu Leu Thr  
1 5 10 15

Ala Val His Leu Gly Gln Cys Val Thr Cys Ser Asp Lys Gln Tyr Leu  
20 25 30

His Asp Gly Gln Cys Cys Asp Leu Cys Gln Pro Gly Ser Arg Leu Thr  
35 40 45

Ser His Cys Thr Ala Leu Glu Lys Thr Gln Cys His Pro Cys Asp Ser  
50 55 60

Gly Glu Phe Ser Ala Gln Trp Asn Arg Glu Ile Arg Cys His Gln His  
65 70 75 80

Arg His Cys Glu Pro Asn Gln Gly Leu Arg Val Lys Lys Glu Gly Thr  
85 90 95

Ala Glu Ser Asp Thr Val Cys Thr Cys Lys Glu Gly Gln His Cys Thr  
100 105 110

Ser Lys Asp Cys Glu Ala Cys Ala Gln His Thr Pro Cys Ile Pro Gly  
115 120 125

Phe Gly Val Met Glu Met ala Thr Glu Thr Thr Asp Thr Val Cys His  
130 135 140

Pro Cys Pro Val Gly Phe Phe Ser Asn Gln Ser Ser Leu Phe Glu Lys  
145 150 155 160

Cys Tyr Pro Trp Thr Ser Cys Glu Asp Lys Asn Leu Glu Val Leu Gln  
165 170 175

Lys Gly Thr Ser Gln Thr Asn Val Ile Cys Gly Leu Lys Ser Arg Met  
180 185 190

Arg Ala Leu Leu Val  
195

## (2) INFORMATION FOR SEQ ID NO:133:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 208 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile  
1 5 10 15

Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp  
20 25 30

Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr  
35 40 45

68

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro  
 50                55                60

Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys  
 65                70                75                80  
 \*

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu  
 85                90                95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr  
 100              105              110

Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu  
 115              120              125

Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg  
 130              135              140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys  
 145              150              155              160

Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln Lys  
 165              170              175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr  
 180              185              190

Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
 195              200              205

## (2) INFORMATION FOR SEQ ID NO:134:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 224 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

Met Gly Ala Gly Ala Thr Gly Arg Ala Met Asp Gly Pro Arg Leu Leu  
 1                5                10                15

Leu Leu Leu Leu Leu Gly Val Ser Leu Gly Gly Ala Lys Glu Ala Cys  
 20              25              30

Pro Thr Gly Leu Tyr Thr His Ser Gly Glu Cys Cys Lys Ala Cys Asn  
 35              40              45

Leu Gly Glu Gly Val Ala Gln Pro Cys Gly Ala Asn Gln Thr Val Cys  
50 55 60

Glu Pro Cys Leu Asp Ser Val Thr Phe Ser Asp Val Val Ser Ala Thr  
65 70 75 80

Glu Pro Cys Lys Pro Cys Thr Glu Cys Val Gly Leu Gln Ser Met Ser  
85 90 95

Ala Pro Cys Val Glu Ala Asp Asp Ala Val Cys Arg Cys Ala Tyr Gly  
100 105 110

Tyr Tyr Gln Asp Glu Thr Thr Gly Arg Cys Glu Ala Cys Arg Val Cys  
115 120 125

Glu Ala Gly Ser Gly Leu Val Phe Ser Cys Gln Asp Lys Gln Asn Thr  
130 135 140

Val Cys Glu Glu Cys Pro Asp Gly Thr Tyr Ser Asp Glu Ala Asn His  
145 150 155 160

Val Asp Pro Cys Leu Pro Cys Thr Val Cys Glu Asp Thr Glu Arg Gln  
165 170 175

Leu Arg Glu Cys Thr Arg Trp Ala Asp Ala Glu Cys Glu Glu Ile Pro  
180 185 190

Gly Arg Trp Ile Thr Arg Ser Thr Pro Pro Glu Gly Ser Asp Ser Thr  
195 200 205

Ala Pro Ser Thr Gln Glu Pro Glu Ala Pro Pro Glu Gln Asp Leu Ile  
210 215 220

## (2) INFORMATION FOR SEQ ID NO:135:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 205 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

Met Tyr Val Trp Val Gln Gln Pro Thr Ala Phe Leu Leu Leu Gly Leu  
1 5 10 15

Ser Leu Gly Val Thr Val Lys Leu Asn Cys Val Lys Asp Thr Tyr Pro  
20 25 30

70

Ser Gly His Lys Cys Cys Arg Glu Cys Gln Pro Gly His Gly Met Val  
35            40            45

Ser Arg Cys Asp His Thr Arg Asp Thr Val Cys His Pro Cys Glu Pro  
50            55            60

Gly Phe Tyr Asn Glu Ala Val Asn Tyr Asp Thr Cys Lys Gln Cys Thr  
65            70            75            80

Gln Cys Asn His Arg Ser Gly Ser Glu Leu Lys Gln Asn Cys Thr Pro  
85            90            95

Thr Glu Asp Thr Val Cys Gln Cys Arg Pro Gly Thr Gln Pro Arg Gln  
100           105           110

Asp Ser Ser His Lys Leu Gly Val Asp Cys Val Pro Cys Pro Pro Gly  
115           120           125

His Phe Ser Pro Gly Ser Asn Gln Ala Cys Lys Pro Trp Thr Asn Cys  
130           135           140

Thr Leu Ser Gly Lys Gln Ile Arg His Pro Ala Ser Asn Ser Leu Asp  
145           150           155           160

Thr Val Cys Glu Asp Arg Ser Leu Leu Ala Thr Leu Leu Trp Glu Thr  
165           170           175

Gln Arg Thr Thr Phe Arg Pro Thr Thr Val Pro Ser Thr Thr Val Trp  
180           185           190

Pro Arg Thr Ser Gln Leu Pro Ser Thr Pro Thr Leu Val  
195           200           205

(2) INFORMATION FOR SEQ ID NO:136:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 191 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

Met Gly Asn Asn Cys Tyr Asn Val Val Val Ile Val Leu Leu Val  
1            5            10            15

71

Gly Cys Glu Lys Val Gly Ala Val Gln Asn Ser Cys Asp Asn Cys Gln  
20                25                30

Pro Gly Thr Phe Cys Arg Lys Tyr Asn Pro Val Cys Lys Ser Cys Pro  
35                40                45

Pro Ser Thr Phe Ser Ser Ile Gly Gly Gln Pro Asn Cys Asn Ile Cys  
50                55                60

Arg Val Cys Ala Gly Tyr Phe Arg Phe Lys Lys Phe Cys Ser Ser Thr  
65                70                75                80

His Asn Ala Glu Cys Glu Cys Ile Glu Gly Phe His Cys Leu Gly Pro  
85                90                95

Gln Cys Thr Arg Cys Glu Lys Asp Cys Arg Pro Gly Gln Glu Leu Thr.  
100                105                110

Lys Gln Gly Cys Lys Thr Cys Ser Leu Gly Thr Phe Asn Asp Gln Asn  
115                120                125

Gly Thr Gly Val Cys Arg Pro Trp Thr Asn Cys Ser Leu Asp Gly Arg  
130                135                140

Ser Val Leu Lys Thr Gly Thr Thr Glu Lys Asp Val Val Cys Gly Pro  
145                150                155                160

Pro Val Val Ser Phe Ser Pro Ser Thr Thr Ile Ser Val Thr Pro Glu  
165                170                175

Gly Gly Pro Gly Gly His Ser Leu Gln Val Leu Thr Leu Phe Leu  
180                185                190

## (2) INFORMATION FOR SEQ ID NO:137:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO;137:

TATGGATGAA GAAACCTTCTC ATCAGCTGCT GTGTGATAAAA TGTCCGCCGG GTAC  
54

## (2) INFORMATION FOR SEQ ID NO:138:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 380 amino acids

72

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His  
1 5 10 15

Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His  
20 25 30

Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser  
35 40 45

Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro  
50 55 60

Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His  
65 70 75 80

Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe  
85 90 95

Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala  
100 105 110

Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe  
115 120 125

Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn  
130 135 140

Cys Ser Thr Phe Gly Leu Leu Ile Gln Lys Gly Asn Ala Thr His  
145 150 155 160

Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile  
165 170 175

Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr  
180 185 190

Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly  
195 200 205

Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser  
210 215 220

Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn

73

225            230            235            240  
Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys  
245            250            255  
  
Glu Ser Ser Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu  
260            265            270  
  
Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro  
275            280            285  
  
Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu  
290            295            300  
  
Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr  
305            310            315            320  
  
Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe  
325            330            335  
  
Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His  
340            345            350  
  
Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile  
355            360            365  
  
Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu  
370            375            380

## (2) INFORMATION FOR SEQ ID NO:139:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 380 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His  
1            5            10            15  
  
Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His  
20            25            30  
  
Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr  
35            40            45

Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro  
50 55 60

Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His  
65 70 75 80

Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe  
85 90 95

Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala  
100 105 110

Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe  
115 120 125

Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn  
130 135 140

Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His  
145 150 155 160

Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile  
165 170 175

Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr  
180 185 190

Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly  
195 200 205

Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser  
210 215 220

Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn  
225 230 235 240

Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys  
245 250 255

Glu Asn Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu  
260 265 270

Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala  
275 280 285

Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile  
290 295 300

Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr  
305 310 315 320

Leu Lys Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe

75

325 330 335

Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His  
340 345 350

Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile  
355 360 365

Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu  
370 375 380

## (2) INFORMATION FOR SEQ ID NO:140:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

TGGACCACCC AGAACGTACCT TCATTATGAC 30

## (2) INFORMATION FOR SEQ ID NO:141:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

GTCATAATGA AGGTACTTCT GGGTGGTCCA 30

## (2) INFORMATION FOR SEQ ID NO:142:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

GGACCACCCA GCTTCATTAT GACGAAGAAA C 31

(2) INFORMATION FOR SEQ ID NO:143:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

GTTTCTTCGT CATAATGAAG CTGGGTGGTC C 31

(2) INFORMATION FOR SEQ ID NO:144:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

GTGGACCACC CAGGACGAAG AAACCTCTC 29

(2) INFORMATION FOR SEQ ID NO:145:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:

GAGAGGTTTC TTCGTCCCTGG GTGGTCCAC 29

## (2) INFORMATION FOR SEQ ID NO:146:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
~ (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:

CGTTTCCTCC AAAGTTCCTT CATTATGAC 29

## (2) INFORMATION FOR SEQ ID NO:147:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:

GTCATAATGA AGGAACTTG GAGGAAACG 29

## (2) INFORMATION FOR SEQ ID NO:148:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:

GGAAACGTTT CCTGCAAAGT ACCTTCATTA TG 32

## (2) INFORMATION FOR SEQ ID NO:149:

78

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:

CATAATGAAG GTACTTGCA GGAAACGTTT CC 32

(2) INFORMATION FOR SEQ ID NO:150:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:

CACGCAAAAG TCGGGAAATAG ATGTCAC 27

(2) INFORMATION FOR SEQ ID NO:151:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:

GTGACATCTA TTCCCGACTT TTGCGTG 27

(2) INFORMATION FOR SEQ ID NO:152:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid

79

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

\*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:

CACCCTGTCG GAAGAGGCCT TCTTC

25

(2) INFORMATION FOR SEQ ID NO:153:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:153:

GAAGAAGGCC TCTTCCGACA GGGTG

25

(2) INFORMATION FOR SEQ ID NO:154:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:

TGACCTCTCG GAAAGCAGCG TGCA

24

(2) INFORMATION FOR SEQ ID NO:155:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

80

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:  
TGCACGCTGC TTTCCGAGAG GTCA 24

(2) INFORMATION FOR SEQ ID NO:156:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:  
CCTCGAAATC GAGCGAGCAG CTCC 24

(2) INFORMATION FOR SEQ ID NO:157:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:  
CGATTTCGAG GTCTTCTCG TTCTC 25

(2) INFORMATION FOR SEQ ID NO:158:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:

CCGTGAAAAT AAGCTCGTTA TAACTAGGAA TGG

33

(2) INFORMATION FOR SEQ ID NO:159:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:

CCATTTCCTAG TTATAACGAG CTTATTTCA CGG

33

(2) INFORMATION FOR SEQ ID NO:160:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:160:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:161:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:161:

82

CCTCTCTCGA GTCAGGTGAC ATCTATTCCA CACTTTGCG TGGC

44

## (2) INFORMATION FOR SEQ ID NO:162:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:162:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:163:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:163:

CCTCTCTCGA GTCAAGGAAC AGCAAACCTG AAGAAGGC

38

## (2) INFORMATION FOR SEQ ID NO:164:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:164:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:165:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:165:

CCTCTCTCGA GTCACTCTGT GGTGAGGTTC GAGTGGCC

38

## (2) INFORMATION FOR SEQ ID NO:166:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:166:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

## (2) INFORMATION FOR SEQ ID NO:167:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:167:

CCTCTCTCGA GTCAGGATGT TTTCAAGTGC TTGAGGGC

38

## (2) INFORMATION FOR SEQ ID NO:168:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids

84

(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein  
x

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:168:

Met Lys His His His His His His Ala Ser Val Asn Ala Leu Glu  
1 5 10 15

## INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 00/18667

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/17 A61K38/20 A61K38/55 A61K47/48 A61P19/00  
 A61P25/00 A61P29/00 A61P37/00 //C07K14/705,  
 C12N15/12, (A61K38/17,38:20), (A61K38/17,38:55), (A61K38/17,38:20),

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE, SCISEARCH, EMBASE, CANCERLIT

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 784 093 A (AMGEN INC) 16 July 1997 (1997-07-16) cited in the application  abstract page 12, line 41 - line 49; claims 1-60 ---	1,2, 6-13, 17-25, 30-42
A	ROODMAN G D: "ROLE OF CYTOKINES IN THE REGULATION OF BONE RESORPTION" CALCIFIED TISSUE INTERNATIONAL, NEW YORK, NY, US, vol. 53, no. SUPPL. 01, 1993, pages S94-S98, XP000940463 ISSN: 0171-967X page S95, left-hand column, paragraph 3 -right-hand column, paragraph 1 --- -/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## ° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

24 January 2001

Date of mailing of the international search report

12.07.2001

Name and mailing address of the ISA

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Authorized officer

NIEMANN, F

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 00/18667

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 38:55 , (A61K38/20,38:55)

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 21946 A (RUSSELL DEBORAH A ; THOMPSON ROBERT C (US); SYNERGEN INC (US)) 11 November 1993 (1993-11-11) abstract claims 1-15 --- MARTIN T J ET AL: "Interleukins in the control of osteoclast differentiation." CRITICAL REVIEWS IN EUKARYOTIC GENE EXPRESSION, (1998) 8 (2) 107-23. REF: 143, XP000979080 the whole document -----	
A		

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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24 January 2001

Name and mailing address of the ISA  
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Authorized officer

**NIEMANN, F**

## INTERNATIONAL SEARCH REPORT

ntional application No.  
PCT/US 00/18667

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
**Although claims 1,2,6-13,17-25,30-42 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.**
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see further information sheet invention 1.

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,2,39-42 (partially), 6-13,17-25,30-38

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with TNF-alpha inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with TNF-alpha inhibitors and IL-1 inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with TNF-alpha inhibitors and serine protease inhibitors.

2. Claims: 1,2,39-42 (partially) 14-16, 26-29

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors and IL-1 inhibitors.

3. Claims: 1,2 (partially),3-5

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors.

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with serine protease inhibitors and IL-1 inhibitors.

4. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with IL-6 inhibitors.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with IL-8 inhibitors.

6. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with IL-18 inhibitors.

7. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with ICE modulators.

8. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with FGF-1 to FGF-10, FGF modulators.

9. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with PAF antagonists.

10. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with MMP modulators.

11. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with NOS modulators.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

12. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with modulators of glucocorticoid receptor.

13. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with modulators of glutamate receptor.

14. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with modulators of LPS level.

15. Claims: 1,2 (partially)

a method for treating conditions leading to bone loss, which comprises administering a therapeutically effective amount of a purified and isolated OPG protein in conjunction with noradrenaline or modulators and mimetics thereof.

16. Claims: 43-48

a method of treating an IL-1 mediated disease, which comprises administering therapeutically effective amounts of an IL-1 inhibitor and a serine protease inhibitor

17. Claims: 49-57

a method of treating an TNF-mediated disease, which comprises administering therapeutically effective amounts of a TNF-alpha inhibitor and a serine protease inhibitor

18. Claims: 58-61

a method of treating inflammation, rheumatoid arthritis, SLE, GvHD, which comprises administering an IL-18 inhibitor, a TNF-alpha inhibitor, and an IL-1 inhibitor

## INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 99/18667

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0784093	A 16-07-1997	AU 710587	B	23-09-1999
		AU 1468697	A	17-07-1997
		BG 101813	A	30-09-1998
		CA 2210467	A	03-07-1997
		CN 1182452	A	20-05-1998
		CZ 9702538	A	17-03-1999
		DE 19654610	A	26-06-1997
		EP 0870023	A	14-10-1998
		FR 2742767	A	27-06-1997
		GB 2312899	A,B	12-11-1997
		HU 9801122	A	28-08-1998
		JP 11503616	T	30-03-1999
		NO 973699	A	21-10-1997
		NZ 326579	A	28-01-1999
		PL 321938	A	05-01-1998
		SK 110797	A	12-07-1999
		TR 970550	A	21-07-1997
		WO 9723614	A	03-07-1997
		US 6015938	A	18-01-2000
-----	-----	-----	-----	-----
WO 9321946	A 11-11-1993	AT 188610	T	15-01-2000
		AU 4229493	A	29-11-1993
		CA 2118119	A	11-11-1993
		DE 69327582	D	17-02-2000
		DE 69327582	T	03-08-2000
		DK 639079	T	13-06-2000
		EP 0639079	A	22-02-1995
		ES 2142341	T	16-04-2000
		GR 3033144	T	31-08-2000
		JP 7509223	T	12-10-1995
		PT 639079	T	28-04-2000
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